Quantitative Aspects of Plasma Membrane-Associated Immunoglobulin in Clones of Diploid Human Lymphocytes

Abstract. *The amount of κ and λ chains and Fc fragment associated with the plasma membrane and in the entire cell has been measured for eight lines of human lymphocytes and 21 clones derived from two diploid lines. There was considerable variation in the nature and amount of membrane-associated immunoglobulin and total immunoglobulin among different cell lines and clones. Cells with different phenotypes for membrane-associated immunoglobulin were utilized to show that it is not simply absorbed from the culture medium onto the plasma membrane. The methods are useful for selecting variants which lack or have altered membrane-associated immunoglobulin.*

The nature of membrane-associated molecules that enable cells to react to their environment is a central issue in biology. In the immune system the problem can be clearly delineated because a single molecular species, the immunogen, is capable of inducing cellular proliferation in a limited number of reactive cells. Considerations based largely on the prerequisite for specificity in this process have led to the assumption that the immunogen receptor on the lymphocytic cell surface is the immunoglobulin (Ig) molecule itself (*1*). Evidence for the existence of membrane-associated Ig has been obtained by immunofluorescence or rosette formation (*2*). A functional role for membrane-associated Ig in the induction of an immune response was suggested by the studies of Lesley and Dutton in which prior incubation of spleen cells with heterologous antiserum to L chains and complement inhibited induction of an immune response to red blood cells in vitro (*3*). Association of Ig with cell membrane did not seem to be an obligatory concomitant of Ig secretion since some immunoglobulin secreting plasma cells did not appear to have membrane-associated Ig (*4*).

In our study an antigen-binding method has been used to determine the nature and the amount of membrane-associated Ig in a number of both uncloned and cloned continuous cultures of diploid human lymphocytes. An estimate of the number of membrane-associated molecules per cell and the variation in the nature and number of Ig molecules among different lymphocytes were obtained. A similar analysis of human peripheral lymphocytes has been reported (*5*).

Continuously growing cultures of human lymphocytes were maintained in this laboratory as described (*6*). The morphologic characteristics and mean cell diameter of cells from each line were determined by examination of electron micrographs. Since the immunogobulin content of cultured lymphocytes differs in each phase of the cell cycle, all studies were carried out on random cells in the exponential phase of growth (*6*, *7*). Clones were obtained by the method of Coffino *et al.* (*8*), except that diploid human fibroblasts (W_i38) were used as a feeder layer. Determinations of total cellular immunoglobulin and the amount on the cell surface were based on the Farr antigen-binding capacity (ABC) test as modified for immunoglobulin G (IgG) fragments by Cerottini (*9*). Between 5.0×10^5 and 2.0×10^7 viable lymphocytes were washed in 10 ml of cold (4°C) Earle's spinner salts and suspended in 1.0 ml of spinner salts containing an amount of rabbit antibody to human IgG which precipitated 50 percent of 1.0×10^{-9} g of labeled antigen. Cells and antibody were incubated at 4°C for 60 minutes, and the cells were removed by centrifugation at 800*g* for 10 minutes. The percentage of inhibition by intact cells of the capacity of the rabbit antibody to human IgG to combine with labeled κ and λ chains and Fc fragments was determined. In control incubations where rabbit serums containing antibody to human IgG was replaced by normal rabbit serum, no material was released from the cells which inhibited the capacity of this antibody to combine with the labeled antigens. For determination of the total cellular content of κ or λ chains and Fc fragments, 5.0×10^7 exponentially growing cells were washed three times in 10 ml of Earle's spinner salts and lysed with the detergent NP-40. At the concentration used (0.5 percent), NP-40 did not inhibit the capacity of rabbit antibody to human IgG to bind to any of the antigens studied. The amount of IgG fragment in cells was the same regardless of whether they were disrupted by NP-40 alone, NP-40 and deoxycholate, or sonication. The amounts of κ and λ chains and Fc fragments are expressed as 10^{-9} g of IgG equivalents per 1.0×10^6 cells (*10*). Standard curves for inhibition by a pool of human IgG of rabbit antibody to human IgG binding to κ and λ chains and Fc fragments are illustrated in Fig. 1. It was possible to detect as little as 1.0×10^{-8} g of κ and λ chains and Fc fragments. There was considerable variation in the nature and amount of κ an λ chains and Fc fragments among different cell lines (Table 1). In one cell line (RAJi), no membrane-associated Ig was detected. In two cell lines (IM-10 and 4265), there was excess Fc fragment in the entire cell but not on the surface of the cells. If only intact IgG molecules were bound to the surface membrane and all determinants of an IgG molecule on the cell membrane were equally available to the antiserum, then the ratio of IgG equivalents determined with light chains and Fc fragments should be one. The fact that there was always an excess of κ chains on the surface suggests that either the κ portions of intact IgG molecules are preferentially exposed or that an excess of free light chains can be held on the plasma membrane regardless of the intact IgG molecule. Because other heavy chains were not looked for in our study, it remains possible that some membrane-associated light chains are associated with other heavy chains and therefore are not free. Also, we cannot exclude the possibility that a portion of the Fc fragment is "buried" in the membrane and thus is not available to antiserum. In this regard, it is of interest that in two cell lines (IM-10 and 4265) where there was an excess

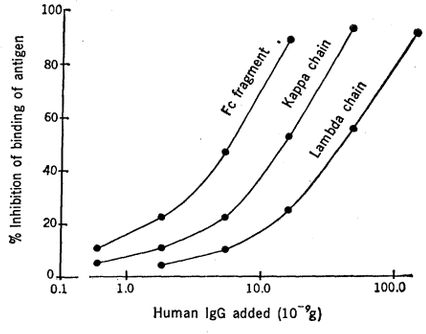

Fig. 1. Inhibition of the binding of rabbit antibody against human IgG to antigen (κ, λ, or Fc). The amount of antigen used was 6.3×10^{-9} g. The amount of inhibitor added varied from 0.1 to 100×10^{-9} g of human IgG (pooled).

Table 1. Membrane-associated and total IgG equivalents for a number of uncloned cells. The results are expressed as equivalents of 10^{-9} g of IgG per 10^6 cells (*10*); Neg., negative; N.D., not done.

Cells	Membrane				Total				Total/membrane		
	κ chain	λ chain	Fc	Light chain/Fc	κ chain	λ chain	Fc	Light chain/Fc	κ chain	λ chain	Fc
WIL_2	5.8	Neg.	Neg.		186	Neg.	Neg.		32		
8866	33.8	Neg.	12.9	2.6	1504	Neg.	388	3.9	44		30
IM1	31.1	Neg.	Neg.		963	Neg.	Neg.		31		
IM-10	1.4	Neg.	0.9	1.5	32	Neg.	78	0.4	23		84
RAJi	Neg.	Neg.	Neg.		Neg.	Neg.	Neg.				
4265	5.1	Neg.	2.3	2.2	77	Neg.	96	0.8	15		42
SS-5	N.D.	N.D.	7.9		Neg.	1010	174	5.8			22
1788	Neg.	76	Neg.		Neg.	1291	Neg.			17	

of Fc fragment in the cells, an excess of κ chains was still associated with the plasma membrane. The ratio of intracellular to membrane-associated light chains was as little as 15 to 1 and as great as 30 to 1.

One difficulty with all previous attempts to measure membrane-associated Ig was that absorption of IgG from serum onto plasma membranes could not be ruled out. In our study we attempted to eliminate the possibility that membrane-associated Ig simply represented secreted Ig molecules reabsorbed to the plasma membrane. WIL_2 and 8866 cells, which have respective phenotypes for membrane-associated Ig of κ + Fc − and κ + Fc +, were grown for 2 days, harvested, and resuspended for 24 hours in medium in which the other cell line was grown (for example, WIL_2 cells into 8866 medium). The phenotype of membrane-associated Ig remained the same for each cell line; thus absorption of secreted molecules onto plasma membranes seemed unlikely. Our results do not rule out the unlikely possibility that among cell lines there are differences in plasma membrane sites which selectively adsorb particular Ig subunits.

In order to determine the degree of variability of Ig synthesis among the cultured cells, clones were obtained and studied; 1.0×10^3 cells were plated in 0.22 percent agarose over a confluent feeder layer of W_i38 cells. After approximately 21 days, clones were "picked" and grown to mass culture for quanitative measurements. Twenty-one clones of WIL_2 and 8866 were studied, and none were found which did not synthesize membrane-associated Ig. This suggests that every cell in these two continuously growing populations was capable of synthesizing membrane-associated Ig and supports the proposition that those cells found negative by fluorescent microscopy were simply in a phase of the cell cycle at which Ig is not synthesized (*6*, *7*). There was considerable variation among different clones in the absolute amounts of total Ig and membrane-associated Ig (Table 2). The amount of the latter could vary fivefold, whereas total Ig varied as much as tenfold among different clones. Differences were also noted in the ratios of κ to Fc and total to membrane Ig fragments. The fact that ratios of membrane-associated κ chains to Fc fragments in 8866 clones were not always the same as total ratios suggests that IgG molecules associate with the plasma membrane in a specific way and are not simply a random percentage of total cellular IgG. These differences were not due to errors in the methods since, when individual clones were again studied, the amount of Ig fragments varied by only ± 10 percent. In the 8866 clones where both κ and Fc fragments were present, there was always an excess of κ chain exposed on the membrane and in the cell.

The amount of IgG on these lymphocyte surfaces may be compared to the amounts of specific antigen receptors in other systems. A clone of WIL cells containing 4.6×10^{-9} g of IgG equivalents on the surfaces of 10^6 cells would have 1.8×10^4 IgG molecules per cell surface. This calculation agrees with those of Byrt and Ada who determined that a single reactive lymphocyte could bind 1.7×10^4 molecules of flagellar antigen (*11*). However, since the combining ratio of membrane-associated Ig for any antigen cannot be known, our studies offer a more direct approach to the quantitation of membrane-associated Ig molecules. The finding that some clones of cells have only κ chains on the surface suggests that light chains may be associated with the plasma membrane in the absence of heavy chains, but further studies will be necessary to exclude the possibility that other heavy chains are present.

Table 2. Membrane-associated and total IgG equivalents for a number of cloned cells. The results are expressed as equivalents of 10^{-9} of IgG per 10^6 cloned cells; Neg., negative.

	Membrane			Total			Total/membrane	
	κ chain	Fc	κ/Fc	κ chain	Fc	κ/Fc	κ chain	Fc
				Clone WIL_2				
2A	4.6	Neg.		88	Neg.		19	
3A	5.7	Neg.		121	Neg.		21	
4A	4.9	Neg.		108	Neg.		22	
5A	5.8	Neg.		76	Neg.		13	
6A	4.1	Neg.		59	Neg.		14	
7A	6.8	Neg.		151	Neg.		22	
				Clone 8866				
1A	68	24	2.4	1571	1083	1.5	23	46
2A	69	27	2.9	2289	1039	2.2	33	39
3A	38	10	3.6	1341	223	6.0	35	21
4A	28	11	2.6	457	161	2.8	17	15
5A	71	25	2.8	1852	503	3.7	26	20
6A	53	22	2.4	2253	438	5.1	43	20
7A	27	11	2.5	581	241	2.4	21	23
8A	50	34	1.5	1239	717	1.7	25	21
9A	61	33	1.8	902	368	2.5	15	11
10A	56	14	4.0	559	259	2.2	10	18
11A	68	15	4.6	963	566	1.7	14	39
12A	55	15	3.6	439	307	1.4	8	20
13A	52	20	2.6	644	302	2.1	12	15
14A	90	39	2.3	1489	730	2.0	17	19
15A	51	24	2.1	1776	1015	1.7	35	42

Our method should be useful selecting variants lacking membrane-associated Ig or in characterizing the structure of membrane-associated Ig subunits.

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Teratogenic Effects of a Chelating Agent and Their Prevention by Zinc

Abstract. *Ingestion of a chelating agent (ethylenediaminetetraacetic acid) by female rats during pregnancy impaired reproduction and resulted in congenitally malformed young. When ethylenediaminetetraacetic acid was fed from days 6 to 21 of gestation, all of the full-term young had gross congenital malformations. These effects were prevented by simultaneous supplementation with 1000 parts per million of dietary zinc.*

Increased use of metal-binding substances in medicine (*1*) has stimulated interest in the potential toxic effects of ethylenediaminetetraacetic acid (EDTA) and related chelating compounds. Recent concern over the proposed use of nitrilotriacetic acid in detergents exemplifies heightened awareness of possible health hazards in widespread use of chelates.

Early evidence of chelate toxicity appeared in 1956, shortly after synthetic chelating compounds became available. Injection of EDTA into pregnant rats resulted in congenital malformations in the young (*2*). Since it was known that this chelating agent binds calcium, the investigators suggested that impairment of fetal development might be due to maternal hypocalcemia (*2*). However, EDTA also complexes zinc (as well as other divalent cations), and the observed teratogenic effects of EDTA may have been due to an induced deficiency of zinc rather than of calcium. Indeed, even a short-term deficiency of dietary zinc during pregnancy has been shown to result in gross congenital malformations in rats (*3*). In contrast, calcium deficiency is not known to be teratogenic even in parathyroidectomized rats (*4*).

In Japanese quail (*Coturnix coturnix japonica*), the addition of small amounts of EDTA improved the hatchability of eggs (*5*, *6*), but higher levels of EDTA reduced hatchability and resulted in some malformations (*5*). In the present investigation, purified diets containing 2 and 3 percent EDTA salts were fed to pregnant rats to determine the effect of dietary EDTA on development of the embryo. The influence of zinc in preventing the effects of EDTA was also tested.

The control ration had the following composition (in percent): soybean protein (*7*), 30.0; sucrose, 57.3; corn oil, 8.0; salt mix (*8*), 4.0; and DL-methionine, 0.7. This ration contained 100 parts per million (ppm) of zinc (provided as zinc carbonate in the salt mix). Crystalline vitamins were given separately (*9*). The EDTA-containing diets were prepared by adding either 2 or 3 g of the disodium salt of EDTA (Na_2EDTA) to 100 g of the control ration (designated as 2 or 3 percent EDTA diets, respectively). Some of the 3 percent EDTA diet was supplemented with additional zinc carbonate, which provided a total content in the diet of 1000 ppm of zinc. The animals had free access to deionized water and food.

Female Sprague-Dawley rats weighing 210 ± 10 g were purchased from a commercial source and were fed the purified control ration for at least 5 days before breeding. Estrous cycles were determined by daily vaginal smears, and the animals were mated overnight with normal stock-fed (*10*)

Table 1. The effect of dietary EDTA supplementation on reproduction in rats. The period of Na_2EDTA ingestion is given as the days of gestation.

Dietary zinc (ppm)	Dietary Na_2EDTA*		Rats (No.)			Implantation sites			Living young at term			
	Percent	Period of ingestion	Mated	With implantation sites	With living young at term	Total No.	Dead or resorbed fetuses (%)	Affected† (%)	Total No.	Mean No. per litter‡	Mean weight of young (g)	Malformed (%)
100	0	0 (controls)	11	11	11	132	6	6	124	11.4	5.3	0
	2	0 to 21	5	5	5	61	5	11	58	11.6	4.6	7
	3	0 to 21	8	0	0	0			0			
		6 to 14	11	10	8	103	40	97	70	7.0	3.7	87
		6 to 21	16	16	11	182	54	100	183	5.5	1.8	100
1000	3	6 to 21	8	7	7	88	8	8	81	11.6	5.0	0

* Percentage is expressed as grams of Na_2EDTA added to 100 g of diet. † Percent of implantation sites having dead, resorbed, or malformed fetuses. ‡ Mean number of rats with implantation sites.