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Immunochemical Studies of Organ and Tumor Lipids XVIII. Cytolipin R Determinants in the Rat Erythrocyte Membrane*

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Summary. Rabbit antisera to rat lymphosarcoma contain antibodies that agglutinate trypsinized rat erythrocytes. These reactions can be specifically inhibited by cytolipin R, a ceramide tetrasaccharide isolated from rat lymphosarcoma. The agglutinin in the rabbit antisera can be absorbed with untreated erythrocytes, showing that cytolipin R determinants are present in the intact rat erythrocyte membrane. Untreated erythrocytes are able to react with antibody, but presumably the number of cytolipin R determinants necessary for agglutination becomes available only after treatment with trypsin. The anti-cytolipin R antibodies in anti-rat lymphosarcoma sera that cause hemagglutination and those that fix complement with this hapten are different, since the agglutinin can be absorbed completely without appreciable decrease in complement-fixing antibody.

Immunization of rabbits with fractions of tissue from another species induces the formation of antibodies, and some of these antibodies may be directed against lipid constituents of the tissue. For reasons that are still unclear, such anti-lipid antibodies arise much more frequently when tumor tissues rather than normal tissues are injected. It has been quite firmly established over the past 10 years that the class of lipids against which such antibodies are usually directed are the neutral glycosphingolipids (Rapport & Graf, 1969). These lipid molecules contain an oligosaccharide chain attached to two hydrophobic residues, a long-chain acid in amide linkage to a long-chain base, called ceramide. One of these substances, which

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has recently been isolated from rat lymphosarcoma, is cytolipin R (Rapport, Schneider & Graf, 1967); although its detailed chemical structure has not yet been fully established, the evidence at hand indicates that the sequence of its six component residues is N-acetylgalactosamine-galactose-galactose-glucose-ceramide.

One of the advantages of working with the cytolipin R system is the ease with which antibodies to cytolipin R are obtained. Immunization of rabbits with particulate fractions of rat lymphosarcoma always provokes the formation of anti-cytolipin R antibodies that can be detected by complement fixation (Rapport & Graf, 1955, 1957). It is the purpose of this communication to show that the availability of these components, namely, antisera to rat lymphosarcoma and pure cytolipin R, permitted us to demonstrate the presence of cytolipin R in the intact rat erythrocyte membrane. We could also show that antibodies which agglutinate rat erythrocytes through their interaction with cytolipin R are different from complement-fixing antibodies and have a higher affinity for cytolipin R than complement-fixing antibodies. Finally, the specificity of this glycosphingolipid-antibody system was examined by comparing the reactions given by many different substances (some very similar to cytolipin R in chemical composition): cytolipin K, cytolipin F, cytolipin H, glucocerebroside, galactocerebroside, galactose-galactose-glucose-ceramide (ceramide trisaccharide), and a mixed ganglioside preparation from normal bovine brain. Cytolipin R, cytolipin K, and cytolipin F (F-hapten) are ceramide tetrasaccharides of very similar composition; they have the same four monosaccharide constituents attached to ceramide, and these are in the same sequence. Their chemical structures are distinctive. Cytolipin K (globoside I) was first isolated from human erythrocytes (Yamakawa, Nishimura & Kamimura, 1965) and human kidney, and cytolipin F was isolated from equine kidney and spleen (Makita, Suzuki & Yosizawa, 1966).

Materials and Methods

Lipids

Cytolipin R was isolated from rat lymphosarcoma according to Rapport *et al.* (1967). Cytolipin K was isolated from human kidney according to Rapport, Graf and Schneider (1964). Cytolipin H was isolated from bovine spleen (Rapport, Schneider & Graf, 1962). Cytolipin F (Forssman hapten) was kindly supplied by Dr. A. Makita. Galactocerebroside was isolated from bovine spinal cord according to Radin, Brown and Lavin (1956). Glucocerebroside was isolated from bovine spleen. Ceramide trisaccharide from human kidney (galactose-galactose-glucose-ceramide) was kindly supplied by Dr. E. Mårtensson. A mixed ganglioside preparation was prepared from bovine grey matter following

the method of Folch and subjecting the product to chromatography on silicic acid to reduce the P content to $<0.1\%$. This material contained the four major ganglioside components of normal human brain: G_1, G_2, G_3, and G_4 (nomenclature of Korey & Gonatas, 1963). Egg lecithin was purchased from Sylvana Chemical Co. (Millburn, N.J.). The neutral glycosphingolipids were all essentially homogeneous by the usual criteria of group analysis and/or thin layer chromatography. It is, of course, well known that fractions homogeneous in their polar moiety (oligosaccharide chain) may appear as two closely related spots on thin layer chromatograms because of relatively small differences in the hydrophobic chains (in either chain length or α-hydroxy substitution).

Enzymes

Crystalline trypsin was purchased from Worthington Biochemical Corp. (Freehold, N.J.), neuraminidase (Type VI from *Clostridium perfringens*) from Sigma Chemical Co. (St. Louis, Mo.) and pronase, B grade, from Calbiochem (Los Angeles, Calif.).

Antisera to Rat Lymphosarcoma

Rabbits (3 to 5 kg) were injected with a particulate fraction of rat lymphosarcoma grown as solid tumor in male Wistar rats. The fraction was prepared as follows. A 3-g portion of minced tissue was homogenized in a Potter homogenizer with 27 ml of 25% sucrose containing 0.002 M $CaCl_2$. The homogenate was filtered through gauze, and the filtrate was layered over 10 ml of 30% sucrose and centrifuged at $800 \times g$ (10 min, 5 °C). The 25% sucrose layer was then centrifuged at $20{,}000 \times g$ (60 min, 5 °C). The sediment was suspended in 30% sucrose using 2 ml/g original tissue and stored frozen. A series of nine injections of particulate material (each representing about 0.5 g tissue) were given intravenously over a 3-week period. Animals were bled by cardiac puncture 4 to 5 days after the last injection. Sera were stored at −20 °C and were inactivated at 57 °C for 30 min before use. Preliminary bleedings from all animals were obtained prior to immunization.

Rat Erythrocytes

Sprague-Dawley or Wistar rats (100 to 300 g either sex) were bled from the aorta into modified Alsever's solution (pH 6.1). Blood from three to four rats was pooled and stored overnight at 4 °C. After filtration through triple gauze to eliminate small clots, the cells were washed three times with saline and diluted as described below.

Enzyme Treatment of Erythrocytes (Boorman & Dodd, 1961)

Trypsin. A stock solution of trypsin containing 10 mg/ml in 0.05 N HCl was stored at 5 °C. This was diluted to 1 mg/ml with 1/15 M phosphate buffer, pH 7.7. To one volume of packed cells, four volumes of the diluted trypsin solution was added, the cell suspension was thoroughly mixed, and then incubated for 40 min at 37 °C with occasional gentle shaking. The cells were centrifuged and washed three times with warm saline.

Neuraminidase. Rat red cells were treated in the same manner as with trypsin, using neuraminidase at a concentration of 0.05 mg/ml and 0.25 mg/ml in 1/15 M phosphate buffer (pH 7.7) containing 5 mM $CaCl_2$.

Pronase. Rat red cells were treated in the same manner as with trypsin, using a double volume of pronase at concentrations of 0.25 mg/ml and 1.00 mg/ml in 1/15 M phosphate buffer (pH 7.7).

Hemagglutination

The test was similar to that described by Boorman and Dodd (1961). Cells were made up as a 4% suspension in saline. Of this suspension, 0.2 ml was added to 0.2 ml diluted antiserum (usually six twofold serial dilutions starting at 1:10 were used). The mixture was kept at 37 °C for 60 min with occasional gentle shaking and was then centrifuged in an International CS centrifuge at $200 \times g$ (1,000 rpm) for 3 min. The sedimented cells were lightly tapped, and the degree of agglutination was recorded on the basis of five steps (4 plus to plus-minus). These results were then converted to a numerical value according to the rating system of Race and Sanger (1958). Values of eight or less were not considered significant.

Hemagglutination-Inhibition

A quantity of pure glycosphingolipid was combined with four parts by weight of lecithin in organic solvent (chloroform or alcohol-chloroform mixtures). The solvent was removed under a stream of nitrogen and the residue was dissolved in 0.1 ml ethanol followed by 0.9 ml saline. Portions of this solution were pipetted into individual tubes, and the volume was brought to 0.45 ml with saline. Then 0.05 ml of undiluted antiserum was added. After incubation for 20 min at 20 °C, these mixtures were diluted serially and tested for hemagglutination.

Absorption with Rat Erythrocytes

To various amounts of packed red cells (from 0.1 to 0.9 ml), 3.0 ml of antiserum at a dilution of 1:5 was added. After thorough mixing, absorption was allowed to proceed at 37 °C for 1 hr with frequent shaking. The cells were then removed by centrifugation at $450 \times g$ (1,500 rpm) for 10 min, and the supernatant solutions tested.

Complement Fixation

The procedure used in this laboratory has been described in detail in earlier publications (Rapport & Graf, 1957*b*; Graf & Rapport, 1960).

Results

Agglutinating Activity of Anti-Lymphosarcoma Sera

Of the 37 anti-lymphosarcoma sera tested, only 10 showed any agglutinating activity with untreated rat erythrocytes, and this activity was of low degree (Fig. 1). In contrast, all 37 antisera showed some degree of reactivity when tested with rat erythrocytes that had been treated with trypsin. The degree of reactivity varied among the antisera (Fig. 1); 27 (73%) gave scores between 16 and 32. Trypsinized rabbit or sheep erythrocytes were not agglutinated.

Effect of Enzyme Treatment of Red Cells

Trypsin treatment of rat erythrocytes for either 30 or 60 min instead of 40 min did not improve the susceptibility of the cells to agglutination by

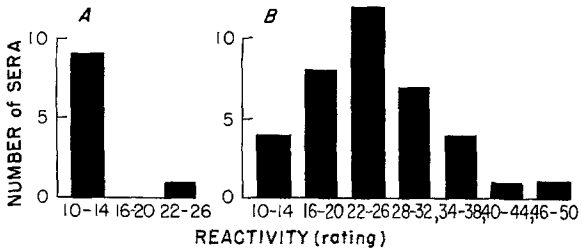

Fig. 1. Agglutination of rat erythrocytes with 37 anti-rat lymphosarcoma sera Panel A: Reactivity of the 10 sera that reacted with untreated cells. Panel B: Reactivity of the 37 sera that reacted with trypsinized cells

these antisera. Neuraminidase and pronase treatment also altered rat erythrocyte membranes to make the cells agglutinable. Using 12 different antisera, a comparison was made of cells treated with trypsin, neuraminidase and pronase. Trypsin treatment produced cells that gave ratings equal to or better than those obtained with pronase in all cases. The differences between scores obtained with neuraminidase-treated and trypsin-treated cells were small. Trypsin treatment appeared to be superior to treatment with 0.05 mg/ml neuraminidase, but equal or inferior to treatment with 0.25 mg/ml neuraminidase.

Inhibition of Agglutination by Glycosphingolipids

Auxiliary lipids. Since it has been established that the interaction of glycosphingolipid haptens with antibody is greatly enhanced by the presence of auxiliary lipids, particularly lecithin, some preliminary experiments were carried out to determine the most effective level. Experiments with two antisera using cytolipin R combined with two-, four-, and sixfold quantities of lecithin showed that fourfold was more effective than twofold, and that very little difference was observed between four- and sixfold quantities. One of these antisera was then studied with varying amounts of cytolipin R combined with fourfold quantities of the following auxiliary lipids: lecithin, cholesterol, lecithin-cholesterol (1:1 w/w), and lecithin-cholesterol (2:1 w/w). Lecithin alone appeared to be decisively superior, and therefore a fourfold quantity of lecithin was used as auxiliary lipid in all subsequent tests.

Specificity

Seven of the best antisera were selected for further study. Eight glycosphingolipids were tested for their capacity to inhibit the agglutination of trypsin-treated erythrocytes by these antisera, using a fourfold quantity of lecithin as auxiliary lipid. In addition, lecithin alone was tested. It was

Table 1. *Hemagglutination-inhibition by glycosphingolipids*[a] *with anti-rat lymphosarcoma sera*

Serum	Quantity of glycosphingolipid for					
	70% inhibition (ng)			50% inhibition (ng)		
	Cyt R	Cyt K	Cyt F	Cyt R	Cyt K	Cyt F
1385	14	>90	>250	7	>90	>250
1387	16	>90	>250	11	30	>250
1386	18	>90	>250	12	28	>250
AS261	18	>90	>250	7	>90	>250
1464	18	>90	>250	12	>90	>250
1408	21	>90	>250	14	24	>250
1457	27	>90	>250	16	>90	>250

[a] Combined with four parts by weight of lecithin as auxiliary lipid. Inhibition with all antisera was below 50% with the largest quantity tested (250 ng) of the following glycosphingolipids: (1) ceramide trisaccharide, (2) cytolipin H, (3) galactocerebroside, (4) glucocerebroside, (5) brain ganglioside mixture, and with 1,000 ng of lecithin.

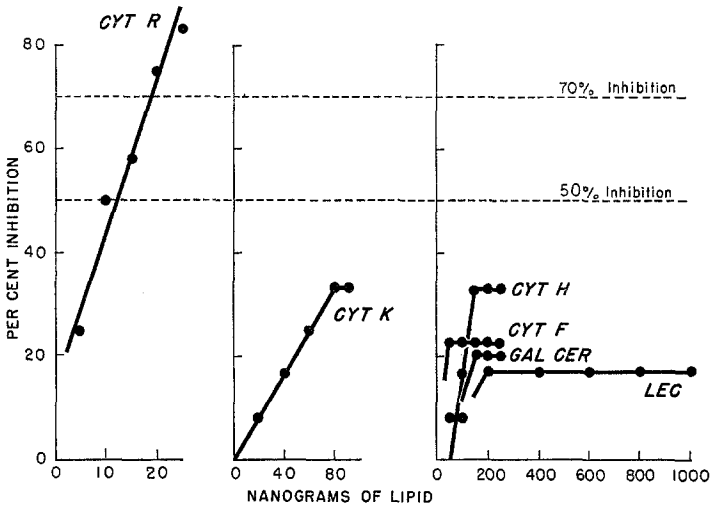

Fig. 2. Relation of degree of inhibition to quantity of inhibitor with antiserum 1464

found that most lipid mixtures were capable of causing a very small degree of inhibition, but this did not increase as the quantity of lipid added was increased. In order to compare the effects of the various lipids, two end points were selected: 70% inhibition and 50% inhibition. The former represents a very substantial effect, the latter a weak effect. The lipids were tested over a range of concentrations, and from a plot of percent inhibition vs. quantity of lipid, the values shown in Table 1 were obtained. Representative plots obtained with one of the antisera are also shown (Fig. 2).

Cytolipin R caused 70% inhibition with all seven antisera in amounts ranging from 14 to 27 ng. None of the other lipids was able to inhibit the reaction to this degree. Cytolipin R caused 50% inhibition in amounts ranging from 7 to 16 ng. With three of the antisera, cytolipin K had this effect in amounts ranging from 24 to 30 ng, but with the other four antisera this degree of inhibition could not be obtained. None of the other lipids was able to cause 50% inhibition. Inspection of the curves in Fig. 2 shows that the weak inhibitory effects level off, so that increases in the quantity of the various lipids do not alter the effects. All seven antisera were studied in this manner. For six of the seven sera, the degree of inhibition caused by the largest quantity of cytolipin R tested was above 80%.

Absorption Studies

Both untreated and trypsin-treated rat red cells were able to absorb the agglutinating antibody completely, but trypsin-treated cells were much more effective (Table 2). Sheep erythrocytes did not absorb the agglutinin.

Antisera from which the hemagglutinin had been absorbed were then tested with cytolipin R in the region of antigen excess (300 and 500 ng of cytolipin R; 6 units of complement) to determine how much complement-fixing antibody to cytolipin R had been removed. In most cases, the quantity of this antibody removed was not significant (less than 8%), and in all cases it was less than 35%. Isofixation curves of four antisera before and after absorption are shown in Fig. 3. Anti-cytolipin R antibody causing agglutination is therefore different from that involved in complement fixation.

Table 2. *Absorption of hemagglutinin by untreated and trypsin-treated erythrocytes*

Antiserum	Volume of packed cells for 95% absorption[a]	
	Untreated cells (ml)	Trypsin-treated cells (ml)
1385	0.65	0.23
1386	0.52	0.17
1387	0.79	0.12
1393	0.98	0.22
1396	0.63	0.19
1408	0.44	0.23
1457	0.91	0.32
1464	0.79	0.22
1678	0.60	0.18

[a] Using 3.0 ml of antiserum at a dilution of 1:5.

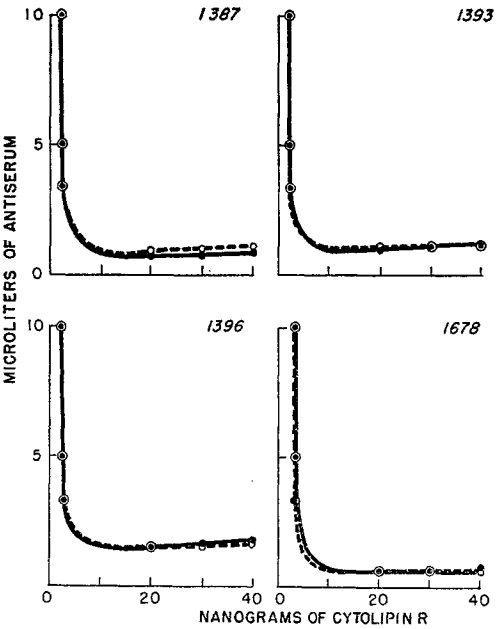

Fig. 3. Isofixation curves of anti-lymphosarcoma sera before and after complete absorption of hemagglutinin with untreated erythrocytes

Discussion

The studies described show that cytolipin R determinants are present in the intact membrane of the rat erythrocyte. The evidence is based on several observations that might, if considered separately, lead to a different conclusion, and it may be of some value to discuss these observations in their logical sequence. First, antibodies prepared by immunizing rabbits with rat erythrocytes are not useful reagents for demonstrating cytolipin R determinants. Although they agglutinate erythrocytes well, the agglutination is not inhibited by cytolipin R (Graf & Rapport, *unpublished observations*). Furthermore, such anti-erythrocyte sera do not give significant reactions with cytolipin R by complement fixation. The antiserum reagent therefore had to be obtained in some way other than immunization with the cell under study, and was in this case obtained by hyperimmunization with a particulate fraction from rat lymphosarcoma. Second, anti-rat lymphosarcoma sera, despite their high titers of complement-fixing antibody directed against cytolipin R, are not good reagents for the demonstration of cytolipin R in the intact membrane; most of these sera agglutinate untreated erythrocytes very weakly or not at all. However, the agglutination is readily observed by pretreating the cells with trypsin. The demonstration

that the agglutination is caused by antibodies directed against cytolipin R determinants is made possible by the availability of pure cytolipin R, which can effectively inhibit the reaction at very low concentrations. The availability of the pure lipid is essential, since the antiserum was prepared against a complex tissue fraction containing many antigens. It is interesting in this regard that antisera prepared against pure cytolipin R by combination immunization with albumin and complete Freund's adjuvant did not contain appreciable hemagglutinin activity despite the presence of anti-cytolipin R antibody detectable by complement fixation. The components of the test system for demonstration of cytolipin R determinants in the intact cell membrane are: (1) trypsin-treated rat erythrocytes, (2) anti-rat lymphosarcoma serum that agglutinates these cells by virtue of cytolipin R determinants in the membrane (established by inhibition experiments with pure cytolipin R), and (3) intact erythrocytes. It is then found that intact rat erythrocytes have the capacity to absorb the antibody that causes agglutination. Cytolipin R is therefore not a cryptic antigen that only becomes exposed after trypsin treatment of the cells. It is available for reaction in the intact membrane. The number of groups that are normally exposed are apparently not sufficient to permit agglutination with the available antisera, but they are able to absorb antibodies from the antisera. Trypsin treatment presumably exposes more of these determinants, since, after such treatment, cells are almost three times more effective in absorbing the agglutinating antibody.

Clearly, the antibodies responsible for hemagglutination are different from those involved in complement fixation. Aside from the observations cited above, it was possible to eliminate all the agglutinating antibody from three antisera without significant reduction of the complement-fixing antibody, and, in one other serum, the complement-fixing antibody level was only partially reduced. The preferential absorption indicates that the agglutinin has a higher affinity for cytolipin R in the membrane than the complement-fixing antibody, and furthermore that the complement-fixing antibody probably does not effectively compete with the agglutinin for cytolipin R sites.

Studies of the alteration of erythrocyte properties through antibodies directed against glycosphingolipid determinants have been reported by Makita *et al.* (1966) and by Koscielak, Hakomori and Jeanloz (1968). Makita *et al.* (1966) studied hemolysis of sheep erythrocytes by rabbit anti-sheep erythrocyte antibody, and observed that the complement-dependent lytic reaction was inhibited by a ceramide tetrasaccharide isolated from equine kidney or spleen (F-hapten or cytolipin F). This system did not

require that the erythrocyte be pretreated with enzyme. Inhibition by cytolipin F was greatly enhanced by auxiliary lipids. These authors also reported that cytolipin F was able to inhibit completely the agglutination of human group A erythrocytes by human anti-A serum. Koscielak *et al.* (1968) studied the agglutination of human erythrocytes by three antisera prepared by immunization of rabbits with bovine serum albumin and globoside I (cytolipin K). Agglutination required pretreatment of the erythrocytes with trypsin. Unfortunately the published data do not permit comparisons, either qualitative or quantitative, with the studies presented here.

As is the case with other immunochemical systems involving lipids and with other biologically active lipids, the auxiliary lipid phenomenon plays a very significant role (Rapport, 1962; Rapport & Graf, 1969). This role may be complex, having components related to dispersibility, the formation of mixed micelles capable of maximal penetration by protein, and/or formation of aggregates with a particular charge. We have dealt with this problem empirically, utilizing the minimal quantity of the simplest auxiliary lipid that will produce the most intense reactions. This permits the most meaningful evaluation of specificity. In these experiments, a fourfold quantity of lecithin appeared best – less produced a less intensive reaction and more did not measurably enhance it. The relevance of these auxiliary lipid effects to activity of glycosphingolipids in natural membranes is speculative at best, and it does not seem worthwhile at the present time to examine such effects exhaustively or to overemphasize their mechanism of action.

It becomes important in attempts to establish the biological significance of these immunochemical systems to evaluate three variables: the distribution of reactivity in different antisera, the specificity of reaction with different glycosphingolipids, and the role of ubiquitous auxiliary lipids such as lecithin and cholesterol. In the earlier studies cited, such information has not been presented. If the reproducibility of these immunochemical systems is not established, then their utility for gaining further information about cell membranes cannot be judged. The studies described in this report have attempted to overcome some of these obstacles. They indicate that useful information about cell membrane structure can be acquired by careful and systematic evaluation of the immunochemical techniques.

Inhibition of agglutination appears to be more specific than complement fixation in detecting the difference between cytolipin R and cytolipin K. Although many anti-lymphosarcoma sera cross-react with cytolipin K by complement fixation, none of the seven sera tested could be inhibited to the extent of 70% of their hemagglutinating activity by cyto-

lipin K. Both complement fixation and hemagglutination inhibition wer equally decisive in distinguishing cytolipin F from cytolipins R and K.

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References

Boorman, K. E., Dodd, B. E. 1961. *In:* An Introduction to Blood Group Serology p. 80. J. and A. Churchill, London.

Graf, L., Rapport, M. M. 1960. Immunochemical studies of organ and tumor lipids. VII The reactivity of anti-human tumor sera with cytolipin H, cardiolipin, and Forssmai haptens. *Cancer Res.* **20**:546.

Korey, S. R., Gonatas, J. 1963. Separation of human brain gangliosides. *Life Sci.* **2**:296

Koscielak, J., Hakomori, S., Jeanloz, R. W. 1968. Glycolipid antigen and its antibody *Immunochemistry* **5**:441.

Makita, A., Suzuki, C., Yosizawa, Z. 1966. Chemical and immunological characteriza tion of the Forssman hapten isolated from equine organs. *J. Biochem. (Tokyo)* **60**:502.

Race, R. R., Sanger, R. 1958. *In:* Blood Groups in Man. p. 246. Blackwell Scientific Publications, Oxford, England.

Radin, N. S., Brown, J. R., Lavin, F. B. 1956. The preparative isolation of cerebrosides *J. Biol. Chem.* **219**:977.

Rapport, M. M. 1962. Lipid-lipid interactions and relation of function to structure of membranes. *In:* Ultrastructure and Metabolism of the Nervous System, vol. 40. S. Korey, A. Pope and E. Robbins, editors. p. 159. Research Publications A.R.N.M.D. Williams and Wilkins, Baltimore.

Rapport, M. M., Graf, L. 1957*a*. Immunochemical studies of organ and tumor lipids. III. The pattern of response of antibody reacting with rat-lymphosarcoma lipids. *Cancer* **10**:438.

— — 1957*b*. Immunochemical analysis based on complement fixation. *Ann. N.Y. Acad. Sci.* **69**:608.

— — 1969. Immunochemical reactions of lipids. *Progr. Allergy* **13**:273.

— — Schneider, H. 1964. Immunochemical studies of organ and tumor lipids. XIII. Isolation of cytolipin K, a glycosphingolipid hapten present in human kidney. *Arch. Biochem. Biophys.* **105**:431.

— Schneider, H., Graf, L. 1962. Immunochemical studies of organ and tumor lipids. XI. A simplified preparation of cytolipin H. *J. Biol. Chem.* **237**:1056.

— — — 1967. Cytolipin R: A pure lipid hapten isolated from rat lymphosarcoma. *Biochim. Biophys. Acta* **137**:409.

Yamakawa, T., Nishimura, S., Kamimura, M.1965. The chemistry of the lipids of post-hemolytic residue or stroma of erythrocytes. XIII. Further studies on human red cell glycolipids. *Jap. J. Exp. Med.* **35**:201.