Immunochemical studies of organ and tumor lipids. XIX. Cytolytic action of antibodies directed against cytolipin R

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Abstract Rabbit antisera to rat lymphosarcoma contain antibodies that are cytolytic for rat erythrocytes in the presence of complement. The reaction can be inhibited completely by pure cytolipin R showing that (*a*) immune hemolysis can be mediated through lipid determinants in the membrane, and (*b*) that cytolipin R determinants are present in the intact erythrocyte membrane and exposed on the surface. Optimal conditions for measurement of cytolysis in this system based on release of ^{51}Cr are described. Degrees of specificity of a number of different antilymphosarcoma sera are shown, based on inhibition of cytolysis by cytolipin R, cytolipin K, cytolipin H, cytolipin F (F-hapten), glucocerebroside, galactocerebroside, ceramide trisaccharide (cer-glu-gal-gal), and a mixed brain ganglioside preparation. The data suggest that cytolytic antibodies and agglutinating antibodies in these antisera are distinctive despite their common specificity for cytolipin R. Lymphosarcoma cells are more effective than erythrocytes in absorbing cytolytic antibodies.

Supplementary key words haptens · rat erythrocytes · hemagglutinin · ^{51}Cr · cytotoxicity · lymphosarcoma · membrane

Cytolipin R is a ceramide tetrasaccharide containing four carbohydrate residues in the linear sequence glucose-galactose-galactose-*N*-acetylgalactosamine (Ref. 1 and unpublished studies). It is therefore very similar in structure to two other naturally occurring ceramide tetrasaccharides, cytolipin K or globoside I (2) and cytolipin F or F-hapten (3), but can be distinguished from them by physical, chemical, and immunological properties. Although cytolipin R was first detected in and isolated from rat lymphosarcoma, it was recently shown that cytolipin R is present in rat erythrocytes (4). This evidence was based on the observation that trypsin-treated erythrocytes could be agglutinated by antisera to rat lymphosarcoma and that the reaction could be completely and specifically inhibited by small quantities of pure cytolipin R. Although untreated erythrocytes were not particularly susceptible to agglutination by these antisera, the presence and availability for reaction of cytolipin R determinants in the intact erythrocyte membrane was deduced from the capacity of such cells to absorb the hemagglutinin.

These studies have now been extended. We wish to describe a cytolytic reaction produced by antibodies directed against cytolipin R determinants, and mediated through these determinants in intact membranes of rat erythrocytes. This reaction is of interest for several reasons. First, it confirms by an independent method the presence and availability for reaction with antibody of cytolipin R determinants in the intact rat erythrocyte membrane. Second, it permits evaluation of the specificity of glycosphingolipid–antibody reactions in a completely new system. And finally, it provides a new model for studying alterations in cell membrane structure through immunochemical reactions involving specific, chemically defined, membrane determinants.

In the studies to be described, the antisera were prepared against rat lymphosarcoma, because such antisera always contain antibodies directed against cytolipin R. The cytolytic reaction is complement-dependent, and the cytolytic activities of these antisera were determined using normal rabbit serum as a source of complement. Antibody specificity to various glycosphingolipids (galactocerebroside, glucocerebroside, cytolipin H, cytolipin K, cytolipin F, cytolipin R, ceramide trisaccharide [cer-glu-gal-gal], and a preparation of mixed brain

gangliosides) was studied by determining the degree of inhibition of cytolysis with different quantities of pure lipid.

MATERIALS AND METHODS

Lipids

Cytolipin R, cytolipin K, and cytolipin H were isolated from rat lymphosarcoma, human kidney, and bovine spleen, respectively (1, 2, 5). Cytolipin F (from equine spleen) was kindly supplied by Dr. A. Makita. Galactocerebroside and glucocerebroside were isolated from bovine spinal cord and bovine spleen, respectively (6). Ceramide trisaccharide (from human kidney) was kindly supplied by Dr. E. Mårtensson. A mixture of gangliosides containing the four main ganglioside components of normal brain was prepared from bovine gray matter and chromatographed on silicic acid columns until the phosphorus content was <0.1%. Lecithin (from egg) was purchased from the Sylvana Chemical Co., Millburn, N.J. The neutral glycosphingolipids were all essentially homogeneous by the usual criteria of group analysis or thin-layer chromatography, or both.

Antisera to rat lymphosarcoma

Rabbits (3–5 kg) were injected with a particulate fraction of rat lymphosarcoma, and antisera were harvested and stored as described in a previous publication (4). They were inactivated at 56°C for 30 min before use. Preliminary blood samples from all animals were obtained prior to immunization.

Rat erythrocytes

Sprague-Dawley rats (100–300 g, male or female) were bled from the aorta, and the blood was collected in an equal volume of Alsever's solution. After filtration through gauze or wire mesh to remove small clots, the cells were collected by centrifugation, washed three times with physiological saline, and immediately labeled with radioactive chromate.

Radioactive chromate

Sodium chromate (^{51}Cr, 100–175 mCi/mg) in buffer solution (pH 8) was purchased from Tracerlab, Waltham, Mass.

Complement

Fresh normal rabbit serum was prepared from rabbit blood obtained by cardiac puncture. It was stored at −20°C until used. Fresh frozen guinea pig serum was obtained commercially (Grand Island Biological Co., Grand Island, N.Y.).

Incubation medium (FF medium)

This was prepared by mixing 34 ml of "Fisher's medium for leukemic cells" (Grand Island Biological Co.) with 6 ml of "fetal calf serum" (same source), and adding several drops of 0.1 N NaOH to bring the pH to 7.0 ± 0.1.

Buffered saline (PBS)

This buffer was prepared from 2.44 g of KH_2PO_4, 8.10 g of Na_2HPO_4, and 4.25 g of NaCl, brought to 1 liter with distilled water.

EDTA-buffered saline

In 1 liter of PBS, 3.92 g of EDTA (disodium salt) was dissolved. The solution was then adjusted to pH 6.9.

Trypsinization of erythrocytes (7)

A stock solution of crystalline trypsin (Worthington Biochemical Corp.) containing 10 mg/ml in 0.05 N HCl was stored at 5°C. This was diluted to 1 mg/ml with M/15 phosphate buffer, pH 7.7. To 1 vol of packed cells 4 vol of the diluted trypsin solution was added, and the cell suspension was 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.

Radiolabeling of rat erythrocytes

To 0.1 ml of packed red cells, 0.5 ml of FF medium was added, and the suspension was incubated with about 100 μCi of radiochromate for 3 hr at 37°C with occasional shaking. The cells were then collected by centrifugation at 1500 rpm for 5 min (CS International centrifuge) and washed three times with 5-ml portions of cold FF medium. A suspension containing 10^7 labeled cells/ml of FF medium was then prepared (by fine adjustment of volume after counting the cells in a rough dilution).

Cytolytic activity

The test was carried out by the methods of Sanderson (8) and Wigzell (9). To 0.1 ml of antiserum in the appropriate dilution, 0.1 ml of complement (usually a 1:20 dilution of fresh-frozen normal rabbit serum) and then 0.1 ml of labeled cells (10^7 cells/ml, in FF medium) were added. The mixture was incubated at ambient temperature (23–25°C) for 80 min with occasional shaking. 0.30 ml of ice-cold EDTA–PBS solution was added to stop the reaction. The mixture was centrifuged immediately for 5 min at 1500 rpm (CS International centrifuge), and 0.30 ml of supernatant solution was carefully removed for counting in an Auto-Gamma

Counter (model 3001, Packard Instrument Co.). Samples were counted for 5- or 10-min periods. Two controls were included: a cell control (giving the total number of counts in the test), which consisted solely of 0.10 ml of cell suspension, and a combined complement and "non-release" control, which contained 0.10 ml of cell suspension, 0.10 ml of complement (normal rabbit serum dilution), and 0.10 ml of FF medium. Percentage cytolysis was then calculated as follows: (counts in reaction tube)/(1/2 counts in cell control) × 100.

Inhibition of cytolysis by cytolipin R and other lipids

For inhibition tests, antisera which produced cytolysis in excess of 95% were selected. A quantity of antiserum was then chosen that would cause about 80% lysis of erythrocytes in the absence of inhibitor. An aqueous solution of sphingolipid plus auxiliary lipid was then prepared as follows. A solution containing 1 μg of sphingolipid and 4 μg of lecithin in organic solvents (ethanol or chloroform–methanol) was evaporated just to dryness in a stream of nitrogen. The residue was then moistened with 0.025 ml of warm absolute ethanol followed by 0.475 ml of saline. To 0.05-ml aliquots of appropriately diluted antiserum, different quantities of inhibitor were added in a volume of 0.10 ml. After incubation at ambient temperature for 30 min, complement and labeled cells were added. Cytolysis was determined as before. For a given quantity of inhibitor, percentage inhibition was calculated as: $100[1 - (Si - Snrc)/(So - Snrc)]$, where *Si*, *So*, and *Snrc* are the counts found in the supernatant solutions of the samples with inhibitor, without inhibitor, and in the complement plus "non-release" control, respectively.

Absorption tests

Erythrocytes were prepared as described. A suspension of rat lymphosarcoma cells was prepared by teasing fresh solid tumor in FF medium. The cells were collected and washed three times with FF medium. Antisera at a 1:10 dilution were absorbed with various amounts of cells. To 0.1 ml of undiluted antiserum, 0.25, 0.50, or 0.75 ml of a 1:5 (v/v) suspension of cells was added, and the volume was brought to 1.0 ml with FF medium. After incubation for 60 min at 37°C with occasional shaking, cells were removed by centrifugation (1500 rpm, 5 min). Residual cytotoxic antibody was determined, and in some instances, complement-fixing antibody as well.

Complement fixation

The method in use in this laboratory has been described in detail in earlier publications (10, 11).

RESULTS

A number of variables had to be evaluated before the cytolysis test could be standardized. These were the range of cytolytic activity in different anti-rat-lymphosarcoma sera, the presence of such activity in normal rabbit sera, the optimal quantity of complement, the most suitable incubation period, and the relationships of these variables to the number of rat erythrocytes.

Cytotoxicity of antilymphosarcoma sera

We studied a total of 27 rabbit antisera to rat lymphosarcoma, all reacting strongly with cytolipin R by complement fixation. These sera all showed some degree of cytolytic activity toward rat erythrocytes in the presence of complement. Using as a measure of activity the quantity of antiserum required for 50% release of ^{51}Cr from 10^6 labeled cells, this quantity was less than 1 μl with 4 antisera; with 14, it was 1–5 μl; with 3, it was 5–10 μl; and with the remaining 6, it was greater than 10 μl. A representative titration (Fig. 1) of one of the more active sera (1678) shows that over 95% cytolysis was obtained with 2.5 μl, and 50% with 0.8 μl. 17 of the 27 antisera showed cytolysis of at least 95% with 20 μl of antiserum. Normal rabbit sera were inactive: none of eight specimens, previously heated at 56°C to remove complement, showed a significant degree of cytotoxicity using 20 μl, the largest quantity tested.

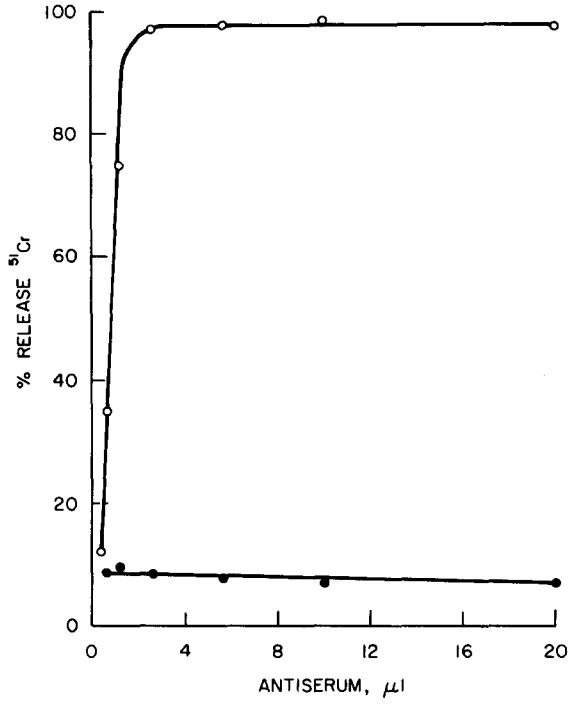

FIG. 1. Representative titration of an antiserum (1678) showing 95% cytolysis of 10^6 rat erythrocytes by 2.5 μl of antiserum, and 50% cytolysis by 0.8 μl, in the presence of complement. Open circles, with antiserum; closed circles, with normal rabbit serum.

Complement requirement

Complement was essential. None of the eight antilymphosarcoma sera tested in the absence of complement was active. To study the dependence on complement activity, individual normal rabbit sera (stored frozen) were diluted in FF medium and incubated with labeled cells and a quantity of antilymphosarcoma serum that gave more than 90% cytolysis. A representative titration (Fig. 2) shows that the maximum effect was produced with 3 μl (0.1 ml of a 1:33 dilution). Five other normal rabbit sera produced this maximal effect with 2.5–10-μl quantities. In the absence of antiserum, none of these six normal rabbit sera had significant activity (i.e., extent of ^{51}Cr release was below 10%). In contrast, a specimen of guinea pig serum (commercial, fresh-frozen, and presumably pooled) produced over 90% cytolysis with 10 μl in the absence of antiserum. For this reason, other specimens of guinea pig serum were not tested. For the experiments reported in this paper, normal rabbit serum was used exclusively as a source of complement, the quantity in each test being double that found by titration (see Fig. 2) to produce maximal activity.

Incubation period

Labeled erythrocytes and complement were incubated for different time periods with three different antisera, using quantities that would produce different degrees of cytolysis: complete, less than 50%, and an intermediate degree. The results (Fig. 3) showed that in all three cases, cytolysis began only after a lag period of about 30–40 min and that the reaction was complete in 80 to 90 min.

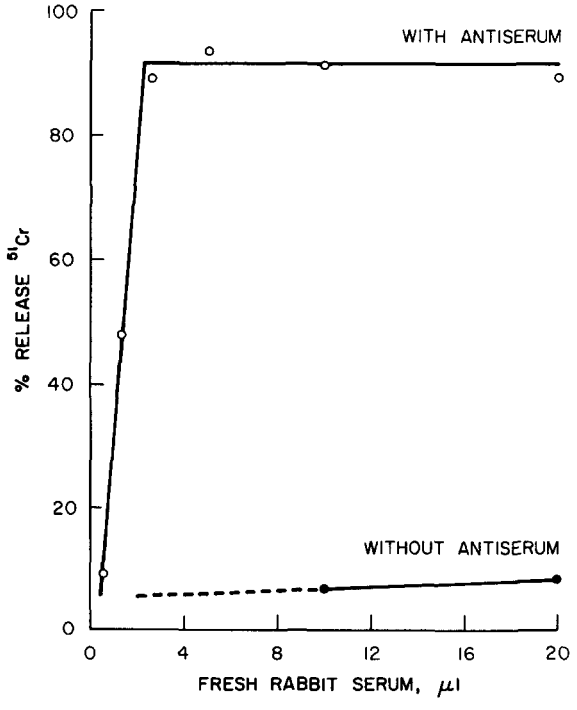

FIG. 2. Complement dependence of the cytolytic system, showing maximum effect produced by 3 μl of fresh rabbit serum. Open circles, with 5 μl of antiserum (1678); closed circles, without antiserum.

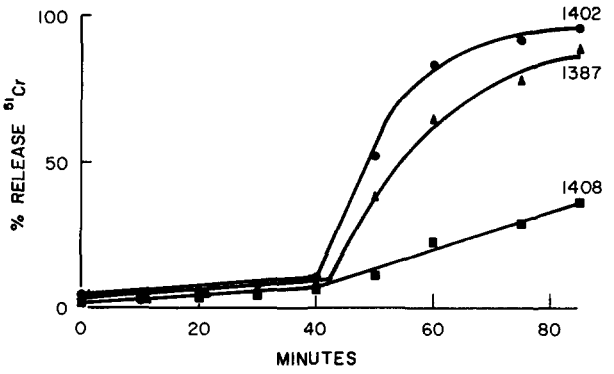

FIG. 3. Time course of the cytolytic reaction with three antisera (10 μl) of different reactivities showing lag period of 30–40 min and completion of reaction in 80–90 min.

Dependence on number of cells

Cytolysis was compared using 10^5, 10^6, and 10^7 erythrocytes and identical quantities of antiserum and complement. With 10^5 cells, blank values (nonrelease control) became appreciable, whereas with 10^7 cells the quantity of antiserum required to produce a given effect was much greater than with 10^6 cells, thereby markedly reducing the sensitivity of the test. The increase in blank values probably indicates cytotoxicity of the normal rabbit sera used as a source of complement.

Inhibition by cytolipin R and other glycosphingolipids

Cytolipin R inhibited the cytolytic activity of antilymphosarcoma sera, and this inhibitory activity was considerably enhanced by addition of lecithin. Using three different antisera, the effects of adding different amounts of lecithin were determined. Ratios of cytolipin R to lecithin (w/w) of 1:1, 1:2, 1:3, and 1:4 were compared. A combination of lipids in which the value was 1:2 was more effective than that with a value of 1:1 and essentially the same as those with values of 1:3 and 1:4. Since the combination with four parts of lecithin was found most effective with hemagglutination inhibition (4), a similar ratio was used in these studies in order to facilitate comparisons of measurements based on the two different techniques.

The various glycosphingolipids, combined with four parts of lecithin by weight, were studied for their inhibitory effects as a function of concentration. A representative dose-response curve with one antiserum is shown (Fig. 4). The relative effects of various lipids can be most readily compared in terms of the percentage inhibition

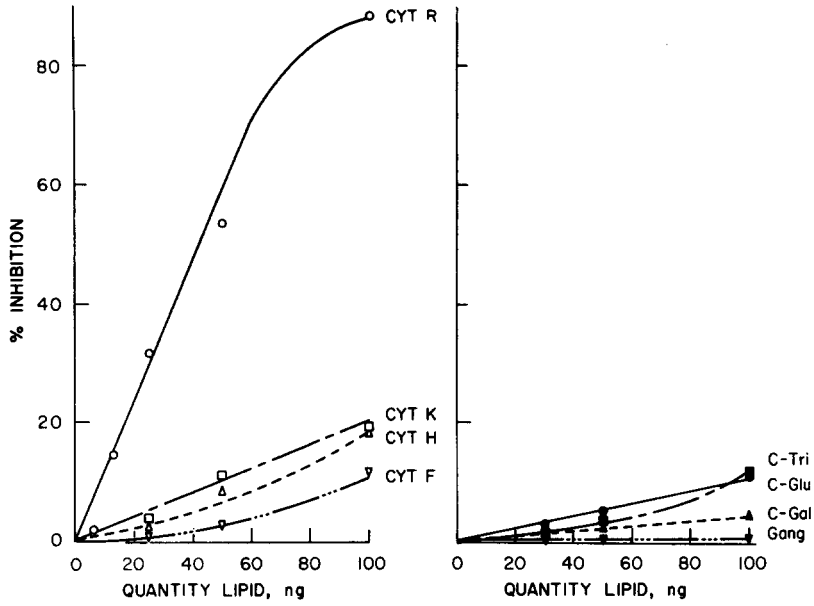

FIG. 4. Representative curves (with one antiserum) showing dependence of the degree of inhibition of cytolysis by cytolipin R and other glycosphingolipids on the quantity of inhibitor. The glycosphingolipids (cytolipin R, cytolipin K, cytolipin H, cytolipin F, ceramide trisaccharide, glucocerebroside, galactocerebroside, and mixed brain gangliosides) were each combined with four parts by weight of lecithin in organic solvent before solution in aqueous medium. In this test, 0.55 μl of antiserum (1393) was used.

obtained with 100-ng quantities of lipid combined with auxiliary lipid (400 ng of lecithin). Results with nine antisera (Table 1) show the specificity of cytolipin R inhibition with respect to other glycosphingolipids and the variability in susceptibility to inhibition by cytolipin R of the cytolytic effects of different antisera.

The cytolytic effects of all nine antisera were effectively inhibited by cytolipin R: six were inhibited 80–89% and three were inhibited 66–79%. The slopes of the dose-response curves suggested that complete inhibition could be attained. The specificity of the antisera showed great variability. Some (e.g., 1386, 1387, 1457) were very specific and did not react appreciably with any lipids other than cytolipin R or cytolipin K. Others (e.g., 1678, 1401, 1456) reacted with other glycosphingolipids as well. However, even with the least specific antisera, only three values in excess of 30% were observed with lipids other than cytolipin R. Two of these values were obtained with antiserum 1456 (32% with cytolipin K, 42% with cytolipin F) and one with antiserum 1401 (34% with cytolipin H). For the most part, antibodies in these less specific antisera showed a greater affinity for cytolipin K, cytolipin F, and cytolipin H than for ganglioside, ceramide trisaccharide, galactocerebroside, or glucocerebroside. Lecithin alone was inactive: with as much as 500 ng, the cytotoxic reactions of only one antiserum

TABLE 1. Inhibition of cytolytic activity of antilymphosarcoma sera with glycosphingolipids and lecithin

Lipid	Antiserum								
	1386	1387	1457	1464	1390	1393	1678	1401	1456
	% inhibition with 100 ng of glycosphingolipid[a]								
Cytolipin R	89	84	66	68	80	89	85	80	79
Cytolipin K	17	26	—	—	20	19	16	26	32
Cytolipin F	—[b]	—	—	13	12	12	19	19	42
Cytolipin H	—	—	—	14	15	20	25	34	27
Ceramide trisaccharide	—	—	—	—	11	12	15	—	—
Galactocerebroside	—	—	—	—	—	—	12	24	15
Glucocerebroside	—	—	—	—	—	11	17	15	14
Ganglioside	—	—	—	—	—	—	11	—	—
Lecithin[c]	—	—	—	—	—	—	—	12	—

[a] Combined with 400 ng of lecithin as auxiliary lipid.
[b] Dashes indicate inhibition below 10%.
[c] 500 ng of lecithin.

TABLE 2. Inhibition of cytolytic activity of antilymphosarcoma sera with different quantities of glycosphingolipids[a]

Antiserum	Wt. of Inhibitor	Lipid Inhibitor: Cytolipin R	Cytolipin K	Cytolipin F	Cytolipin H
	ng		*% inhibition*		
1387	25	22	—[b]	—	—
	50	55	11	—	—
	100	84	26	—	—
1390	25	33	—	—	—
	50	57	—	—	—
	100	80	20	12	15
1678	25	25	—	—	—
	50	56	—	—	10
	100	85	16	19	25
1401	25	33	—	—	—
	50	64	—	11	13
	100	80	26	19	34

[a] Combined with a fourfold quantity (w/w) of lecithin as auxiliary lipid.
[b] Dashes indicate inhibition below 10%.

(1401) were significantly inhibited, and the value was only 12%.

The slopes of the curves relating percentage inhibition to quantity of lipid showed that with some antisera, regions of enhanced specificity may be selected (Table 2). With the four antisera listed, the cytolipin R curve rises more steeply than the curves obtained with cytolipin K, cytolipin F, and cytolipin H; this is seen from the fact that cytolipin R inhibition is more selective with 50 ng than with 100 ng of the various glycosphingolipids.

Mechanism of inhibition

Since cytolipin R (with auxiliary lipid) reacts with these antilymphosarcoma sera to form immune complexes that fix complement, the question may be asked whether inhibition by cytolipin R does not result from removal of complement needed for cytolysis rather than by blocking antibody-combining sites. To answer this question we studied the inhibitory activity of cytolipin R using different quantities of antiserum. Constant amounts of test antigen (50 ng of cytolipin R + 200 ng of lecithin) and complement were mixed with different amounts of antiserum and then incubated with labeled cells. Inhibition of cytolysis was greater than 90% with quantities of antiserum up to 5 μl, the quantity which produced almost maximal cytolysis in the absence of inhibitor. When the quantity of antiserum was increased to 20 μl, inhibition by cytolipin R was rapidly overcome (Fig. 5). This experiment was repeated with two other antisera with the same results. It is therefore clear that cytolipin R combined with cytolytic antibodies and blocked their capacity to react with antigenic sites on the cell membrane. If removal of complement were responsible for the observed inhibition, one would expect that with larger quantities

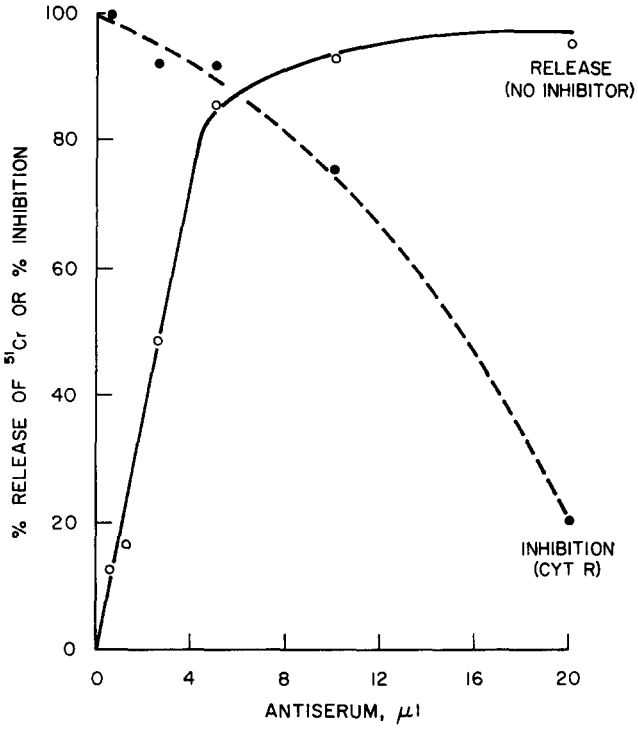

FIG. 5. Dashed line: decrease in the degree of inhibition with increasing quantity of antiserum and constant amount of inhibitor (cytolipin R), showing that inhibition does not result from removal of complement by antibody–inhibitor complex (antiserum 1386; cytolipin R, 50 ng, plus 200 ng of lecithin). Solid line: normal cytolysis curve in the absence of inhibitor.

of antiserum the inhibition would remain at its maximal value, since the formation of more immune complex would be expected to have even larger complement-binding capacity.

Tests with trypsinized erythrocytes

It was found earlier (4) that trypsinization of rat red cells greatly increased their agglutinability by antibodies to cytolipin R as well as their capacity to absorb such antibodies. However, in the cytolytic test, we found that treatment of erythrocytes with trypsin caused them to become sensitive to normal rabbit serum; e.g., 0.1 ml of fresh serum produced 47% hemolysis with 10^6 cells. Neither inactivated antiserum nor inactivated fresh normal rabbit serum caused cytolysis. The results of tests in which the high background (resulting from the use of normal rabbit serum as a source of complement) was taken into consideration appeared to indicate that trypsinized erythrocytes were not more susceptible than untreated cells to immune lysis by antilymphosarcoma sera.

Absorption of cytolytic antibody by erythrocytes and lymphosarcoma cells

Graded absorption of five antisera was carried out with different amounts of erythrocytes and lympho-

sarcoma cells, and the cytolytic antibody remaining in the supernatant solution was assayed. With three of these antisera, over 90% of the antibody was absorbed with either untreated erythrocytes or with lymphosarcoma cells (Fig. 6). With all five antisera, lymphosarcoma cells were more effective than erythrocytes in absorbing cytolytic antibodies. With two of the antisera, no loss in complement-fixing antibody to cytolipin R was detected in specimens from which over 90% of the cytolytic antibody had been absorbed with erythrocytes.

Relation of cytolytic antibody to hemagglutinating antibody

Cytolytic and hemagglutinating activities of antilymphosarcoma sera do not correlate well. This may result from the fact that the test objects are different: cytolytic antibody is tested with untreated rat erythrocytes, whereas hemagglutinating antibody is tested with trypsinized erythrocytes. To determine whether the two types of antibodies were absorbed similarly, three anti-rat-lymphosarcoma sera were partially absorbed using two different quantities of untreated rat erythrocytes, 0.10 ml and 0.30 ml of packed cells per ml of antiserum at a dilution of 1:5. The absorbed sera were then tested for residual cytolytic and hemagglutinating activities. With two of the three antisera, the percentage absorption was very similar in the two tests (30–35% absorption with 0.10 ml of cells, 50–55% absorption with 0.30 ml). With the third antiserum, removal of hemagglutinating antibody was more effective than removal of cytolytic antibody (38% vs. 15% with 0.1 ml of cells, 63% vs. 30% with 0.30 ml). These results suggest that although hemagglutinating antibody and cytolytic antibody may be similar in some antilymphosarcoma sera, they are probably not identical.

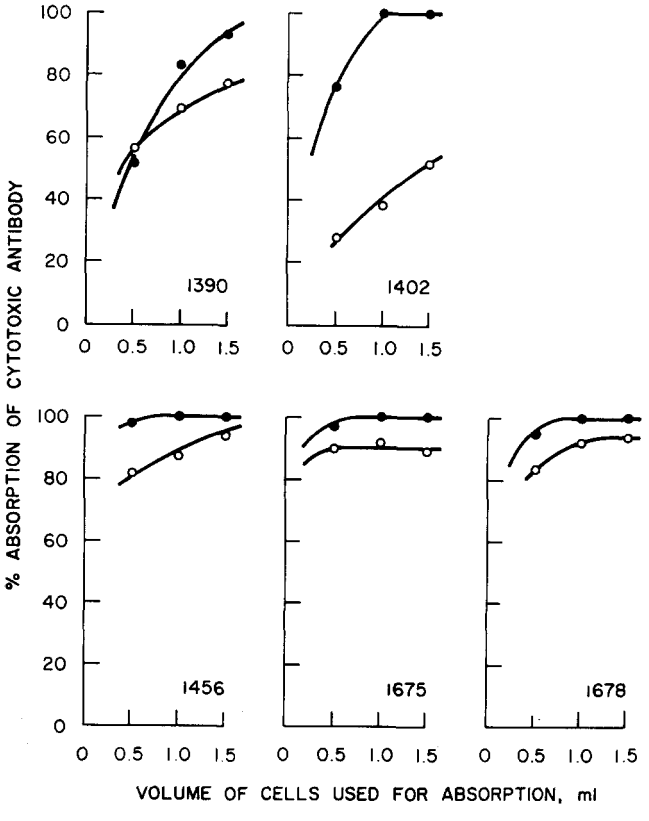

FIG. 6. Absorption of cytotoxic antibody from five antisera, showing that rat lymphosarcoma cells remove antibody more effectively than rat erythrocytes. Closed circles: rat lymphosarcoma cells; open circles: rat erythrocytes.

DISCUSSION

It has now been well established by chemical methods that a variety of glycosphingolipids are present in animal tissues and that different types of cells differ from one another considerably in their glycosphingolipid composition (12, 13). It is equally well established that glycosphingolipid molecules function as antigenic determinants (14), and it appears highly probable that the principal localization of these molecules is in plasma membranes. A major question that now requires attention is the application of immunochemical methods to measure specific determinants in plasma membranes and, in particular, to detect qualitative or quantitative alterations that occur during differentiation or as a result of pathological processes. Two of the most useful quantifiable techniques to measure reactions at the cell surface are agglutination and cytolysis. Because several lines of evidence suggest that cytolipin H bears some relationship to human cancer cells, it has been our principal target for measurement, but the methods we have tried thus far have not been useful. Our studies have led us to examine an alternative model system: antisera to rat lymphosarcoma, in which complement-fixing antibodies to cytolipin R are always to be found, readily agglutinate trypsinized rat erythrocytes (4). The agglutination is mediated through cytolipin R determinants because the reaction can be inhibited almost completely by pure cytolipin R. Although trypsinization of the cells is required for agglutination, untreated erythrocytes can absorb anticytolipin R antibodies, showing that cytolipin R determinants are exposed on the surface of intact cells. The greater effectiveness of trypsinized cells in absorbing the antibody may indicate that more determinants become available for reaction after such treatment.

The cytolysis of rat erythrocytes mediated by antilymphosarcoma sera in the presence of complement is also inhibited almost completely by pure cytolipin R; this reaction therefore confirms that cytolipin R determinants are present and exposed in the intact membrane of rat erythrocytes. Since cytolysis is demonstrable with untreated cells, it is clearly a more sensitive technique

than agglutination for detecting surface antigens. This is consistent with observations with sheep erythrocytes and hemolysin, since it is well known from studies with this classical indicator system for complement fixation that maximal sensitization for immune hemolysis occurs with a higher dilution of antiserum than that causing agglutination (15).

Since the cytolysis reaction is more sensitive than agglutination and requires smaller quantities of antiserum, it is surprising that larger amounts of pure cytolipin R are required to inhibit the reaction. Thus, 70% inhibition of agglutination was produced by 14–27 ng of cytolipin R with seven different antisera, whereas 50 ng were needed to produce 55–64% inhibition of cytolysis (Table 2). This suggests that there are, indeed, differences between the antibodies involved in the two types of reactions. The distinctiveness of antibodies involved in various immunochemical reactions is brought into sharp focus when dealing with a simple monovalent antigen such as cytolipin R. For example, antisera produced by immunizing rabbits with pure cytolipin R and adjuvant, which contain both complement-fixing and precipitating antibodies for cytolipin R, do not show cytotoxicity for rat erythrocytes. What causes the cytotoxic anticytolipin R antibody to arise on immunization of rabbits with particulate fractions of rat lymphosarcoma is still a matter only to be surmised.

The need for larger amounts of pure cytolipin R to inhibit cytolysis (compared with agglutination) also results in lesser specificity of cytolytic antibody compared with agglutinating antibody as judged by the inhibitory activity of other glycosphingolipids. The data in Table 1 show very substantial differences in specificity among nine antisera. Seven of these are appreciably inhibited by cytolipin K, and six by both cytolipin F and cytolipin H. The usefulness of the cytolysis reaction for structural identification of glycosphingolipids is therefore inferior to both agglutination and complement fixation.

Cytolytic reactions mediated by antibodies directed against glycosphingolipid determinants have been reported by Makita, Suzuki, and Yosizawa (3) in a different system. They showed that a pure ceramide tetrasaccharide (cytolipin F, F-hapten), isolated from equine organs, inhibited the hemolysis of sheep erythrocytes by anti-sheep-cell antibody (hemolysin). The applicability of these techniques deserves some discussion. The use of ^{51}Cr-labeling to study cytolysis is, of course, a general method and is applicable to all types of cells. This is the main reason for its use in the present study. Although measurement of hemoglobin release is both more convenient and less expensive, it is restricted to a single type of cell and is more confining with respect to adjustment of the number of cells that may be required for studies with a given antiserum.

The measurement of cytolysis is far from ideal as a method of general applicability. It was found, for example, that normal rabbit sera usually contain cytotoxic antibodies for *mouse* erythrocytes. The occurrence in normal rabbit sera of cytotoxic antibodies for *human* erythrocytes is less frequent, but still presents a significant source of interference. The interference may arise from naturally occurring antibodies in either the antiserum or in the normal serum serving as the source of complement. The degree of interference will depend on the individual systems, since it is clear that very active systems will permit dilutions of both antiserum and complement that will eliminate the naturally occurring interfering factors, whereas weak systems will not. The usefulness of these methods therefore continues to be dependent on the methodical, empirical examination of each system in turn to evaluate its sensitivity, specificity, and reproducibility.

The reactivity of the antibodies in this and the previous study (4) have been interpreted in terms of cytolipin R determinants in the rat erythrocyte membrane. Such an interpretation will retain a degree of uncertainty until the chemical components of this membrane are fully defined chemically, that is, until it can be demonstrated that cross-reacting glycolipids or glycoproteins are absent. However, current evidence, based on both chemical analysis of erythrocyte glycolipids of various species and the specificity of the immunochemical determinants of human erythrocytes that have been most carefully studied, suggests that this degree of uncertainty is rather small.

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