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Review

Structure and specificity of the lipid haptens of animal cells*

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The capacity of proteins and polysaccharides to evoke an antibody response is well established, and there is little difficulty in understanding the structural basis for the reactions of these antigens with specific antibody. In marked contrast, the role of lipids as antigens is still rather ill-defined. Although it is clear that a lipid molecule (cardiolipin) can serve as a determinant of immunological specificity, the number of examples is insufficient to support the formulation of generalizations relating the structure of lipids to their immunological activity. The recent isolation (1, 2) of a second pure lipid with antigenic specificity (cytolipin H) has provided the first model with exactly defined structure, and has demonstrated that certain distinctive features are characteristic of the immunological reactivity of lipids. Since this information permits studies of this type to be pursued systematically, it is worthwhile to review the problem at this time, despite the meagerness of the information at hand. This review will therefore place much greater emphasis on general principles that are important for the immunological study of lipids than on presenting a complete survey of the relevant literature.

Lipids with immunological activity belong to a larger group of cellular substances that have been designated as haptens since they react with antibodies, although they are ineffective in evoking antibody response. It would be difficult to improve on Landsteiner's succinct summary (3*a*) of the state of knowledge concerning cellular haptens despite the passage of more than fifteen years, and, indeed, the far-reaching significance of these substances in animal biology has had no greater champion. It was Landsteiner's view (3*b*) that "there exist two systems of species specificity in the animal kingdom, the specificity of proteins and that of cell haptens. The proteins . . . undergo gradual variation in the course of evolution, while haptens are subject to sudden changes not linked by intermediary stages." The immunological evidence for this point of view lay in "the striking serological differences between the cells of individuals of the same species, the frequent occurrences of so-called heterogenetic antigens, i.e., similarly reacting substances present in unrelated kinds of organisms, and the fact that blood cells of closely related species exhibit . . . much greater differences than the respective serum proteins." The nonprotein nature of cell haptens gave added weight to this thesis. Landsteiner was sensitive to the need for a chemical definition of antigenic structure that would lay a firm foundation for immunological application. It is, of course, not difficult to appreciate the reasons for the lag between chemical and serological analysis of cells, and it is a matter of record that in the past decade at least a dozen new antigenic determinants have been located in the human erythrocyte mosaic without the structure of a single one, new or old, being established. The principal difficulty resides in the fact that these substances are present in low concentrations in animal cells, and major technical problems are associated with separating trace substances from complex mixtures. A second obstacle stems from the fact that a serological method that may be perfectly adequate for investigation of whole cells may prove to be insensitive or otherwise unsuit-

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able when applied to the artifact combinations of substances that result once chemical purification is undertaken.

LIPID HAPTENS: OPERATIONAL DEFINITION

The methodological limitations in studies of haptens are particularly evident when they belong to the class of substances extractable from tissues with nonaqueous solvents. In studies of such substances, ethanol has been the solvent most frequently employed, and extractability with ethanol has been the usual criterion for classifying them as lipids (lipoid, lipid-soluble). This criterion may eventually prove to be reasonably sound, but it is not rigorous; and because nonlipid material may also be extracted, it does not permit the ultimate simplification that is required when mixtures of antigens must be resolved using reagents that are mixtures of antibodies. Antiserum reagents are usually obtained by hyperimmunization (repeated injection) of an animal with foreign cellular material, and antisera specific for a single antigen (or hapten) are not obtained.

One of the principal technical advantages of working with lipids is that the number of immunologically active lipids in a given cell appears to be small, and the water-soluble substances (proteins, polysaccharides) representing most of the antigenic complex can be rapidly eliminated. The introduction by Folch *et al.* (4) of chloroform-methanol extraction of tissues, and the ingenious method for efficiently washing this extract with water, represented a notable advance in the approach to a rigorous definition of the lipid nature of cell haptens by simple, reproducible means. In a comparative study of chloroform-methanol versus alcohol extraction of human tissues, it was found[1] that, with occasional exceptions, both methods gave results in qualitative agreement, although higher titers against antisera were usually obtained with chloroform-methanol extracts. Removal of water-soluble material, for which a convenient procedure is not available for ethanol extracts, will sometimes eliminate most of the reactive substance present in a chloroform-methanol extract, and may be useful in differentiating some haptens. It is clear that these techniques do not provide an entirely satisfactory answer to the question of what constitutes a "lipid" hapten, since a complex molecule may not always be readily classified by such procedures. The achievement here is mainly in the assurance that an active substance which remains consistently in the organic solvent phase will respond to chemical fractionation methods that are useful for lipids in general.

[1] L. Graf and M. M. Rapport, unpublished observations.

MEASUREMENT OF IMMUNOCHEMICAL ACTIVITY OF LIPID HAPTENS

Having defined a "lipid" hapten in operational terms, the next problem to be discussed is their measurement by immunochemical techniques. Several methods are available and have, in fact, been employed by various workers: flocculation, precipitation, complement-fixation, inhibition of cell agglutination or lysis, and, most recently, a combination of precipitation and paper chromatography (5). These methods are not equally serviceable for all purposes. It should be kept in mind that in most immunological studies, antibody estimation has been the goal, and once a suitable formulation of the "test antigen" was achieved, further variations were unnecessary. In contrast, for purification and evaluation of specificity of the antigen, the criterion is the quantity of antigen that reacts with a constant amount of antibody, and experience with most immunological techniques is more limited in this area. Lipids present a special problem because of their limited solubility in aqueous medium, and this problem is magnified because, as will be indicated, lipid antigens may not be active except in the presence of other ("auxiliary") lipids. On the basis of experience with a number of lipid hapten systems—cytolipin H, rat lymphosarcoma hapten, Forssman hapten from horse kidney—the method of complement-fixation appears to be superior to others for following the course of lipid hapten purification. Using rabbit antiserum as the analytical reagent, complement-fixation is a general method for measuring the activity of lipid haptens with sufficient sensitivity to provide consistent results in most instances.[2]

One of the main drawbacks to the convenient application of this method has been overcome with the development of techniques for integrating complement-fixation data into a line curve (isofixation curve) that readily permits relations to be established between quantities of antiserum and antigen that react throughout the zones of antibody and antigen excess, and thus permits systematic comparison of antigens (6). As has been pointed out, satisfactory measurement may require adjustment of the sensitivity to conform to the properties of both antiserum and antigen.

[2] The test of consistency is the comparison of high concentrations of weakly active fractions with lower concentrations of more active fractions.

This sensitivity is determined by the quantity of complement used in the test. The highest level of sensitivity (2 units of complement) is inadvisable because it subjects the system to excessive nonspecific interference. On the other hand, use of too much complement may lower the sensitivity to the point where available antisera are not sufficiently strong to produce a desirable intensity of fixation. It would be difficult to eliminate the abstract quality of these statements without giving concrete examples, and, indeed, immunologists who may not have had extensive experience in antigen purification have unfortunately failed to appreciate the significance of isofixation curves and the need for flexibility in the adjustment of sensitivity level (7).

In attempting to fractionate crude lipids and examine individual fractions for specific activity, two observations that give this kind of problem an individual flavor come to the fore. In a larger sense, these observations, which appear to be general, may have been the chief obstacles to the systematic development of this field. These observations are first, that individual fractions may be intensely anticomplementary (and thus not subject to immunological evaluation), whereas the original crude lipid mixture, in sharp contrast, shows none of this property; and second, that as fractionation proceeds, the total activity recovered markedly decreases, and the intensity of fixation is also diminished. Pangborn's enormous achievement in isolating the first pure lipid hapten, cardiolipin, in the face of these difficulties (8, 9) was of inestimable value in providing a concrete example of how they might be overcome. Pure cardiolipin, a phosphatidic acid, was anticomplementary, and this undesirable property could be eliminated by admixture with lecithin. The combination of lecithin and cardiolipin was not particularly reactive with reagin (human antibody appearing during the early phase of syphilitic infection) but the sensitivity could be increased to almost any desired degree by adding cholesterol. It must be pointed out that the addition of lecithin or cholesterol to "strengthen" haptenic activity of lipid extracts had long been one of the "tricks of the trade" of those engaged in studies of this nature. Their results were, however, strictly empirical since such auxiliary lipids were added to mixtures of undefined content, and in some cases addition of lecithin to different lipid mixtures caused decreased reactivity of some and increased reactivity of others with the same antiserum (10). The demonstration that auxiliary lipids were required with a pure hapten was the first step in appreciating this phenomenon to be a characteristic feature of the reactivity of lipid haptens in complement-fixation. The anticomplementary activity of cardiolipin may have a relation to its acidic properties. Pure cytolipin H, on the other hand, is a neutral molecule and is not anticomplementary; but it too is unreactive in complement-fixation unless combined with auxiliary lipids, a mixture of lecithin and cholesterol being most effective (2). Lecithin and cholestrol may not be unique in enhancing activity of all lipid haptens. Landsteiner and Levene (11) used brain sphingomyelin to increase the serological activity of purified preparations of Forssman hapten. A similar effect was obtained by Papirmeister and Mallette (12) by the addition of phrenosine to a sheep red cell hapten. However, neither pure sphingomyelin nor phrenosine enhanced cytolipin H activity.[3] It will be of considerable interest to determine the extent to which auxiliary functions differ with different pure lipid haptens. The antiserum plays a role here also, and it has been observed in some experiments that changing the relative proportions of auxiliary lipids produces different effects on reactions of the same (pure) hapten with different antisera.[1]

The requirement for auxiliary lipids is most pronounced for *formation of immune complexes that will fix complement*, and, indeed, a quantity of auxiliary lipid that is 50 to 100 times the quantity of pure lipid hapten (cytolipin H)) may be required for maximum effect (2). The role of lipid interactions is not, however, restricted to this phase of immunological activity. Some studies indicate that the *binding of pure lipid hapten with antibody* also requires the presence of other lipids (2, 13). A definitive study of this phenomenon has not yet been made; however, there are reports indicating that lecithin alone is effective with both cardiolipin (13) and cytolipin H;[3] with the latter substance, cholesterol alone is ineffective[3] and does not contribute to the effectiveness of lecithin. The quantity of auxiliary lipid producing a maximum effect in hapten-antibody *binding* may be very much less than that required for *complement-fixation*, and this may in part account for puzzling observations with partially purified lipid fractions which showed activity by absorption methods but not by complement-fixation (14).

Preliminary experiments with cytolipin H[3] suggest that lipid-lipid interactions may also be important in a third aspect of immunological activity, namely, *elicitation of antibody response*. This phenomenon, which is really far too complex to be considered in the same category with the others, has been studied with lipid

[3] M. M. Rapport and L. Graf, unpublished observations.

extracts in the past by the method of "combination immunization," namely, the use of foreign protein (swine serum) as a carrier. The findings in such experiments have been summarized in the last comprehensive review devoted exclusively to lipid haptens (15). However, using limited quantities of pure cytolipin H, it was observed that this method did not evoke antibody response, even when cytolipin H was combined with pure auxiliary lipids.[1] In contrast, antibody to cytolipin H was obtained when a mixture of cytolipin H and crude tissue lipids (and swine serum) was injected.[1] It is not difficult to understand why this phase of the immunological study of lipid substances has always been controversial. With the exception of synthetic lipids and cholesterol, studies in the past have usually been conducted with impure lipids, and evaluation of the final results has included the combined effects of several substances in the three distinct phases of activity mentioned above. We are not yet in a position to understand the bizarre observation of Levene *et al.* (16) that an antiserum to lecithin gave preferential reactions with cholesterol; when a sufficient array of natural haptens is available for study, such strange results may be found to appear so infrequently that they will not require emphasis. It is perhaps relevant here to recall that injection of tissue particulates, in which phosphatides and cholesterol are major lipid constituents and lipid haptens are present in only minor amounts, does not lead to the production of antibody that reacts specifically either with phosphatides or cholesterol.

CHROMATOGRAPHIC PURIFICATION OF LIPID HAPTENS

The technological advance represented by column chromatography on silicic acid for the separation of complex lipids is the single development offering the most promise for revitalizing this area of immunological exploration. With the exception of Pangborn's brilliant effort in the 1940's, this field of activity has been strangely silent since its weary protagonists abandoned the struggle almost a quarter of a century ago. With characteristic insight, Landsteiner had already perceived the new direction in which to move (3*c*). It is a matter of record that the application of this new tool to an old field was begun even in the process of shaping the tool (17). A study of the resolution of phospholipids on silicic acid, started in 1953 in an effort to separate various lipids with thromboplastic activity, was enlarged, later that year, to include the separation of the lipid hapten of rat lymphosarcoma. To date, the major achievement resulting from the use of this new method is the isolation of cytolipin H from human tissues. However, within the past year, studies of other systems have provided strong indications that a systematic method of general applicability may be at hand, whose sole limitations will be the concentration of a given hapten, the number of chemically similar haptens present in a particular source, and the structural complexity of the molecules themselves. Whether a direct approach to purification and characterization of lipid haptens is attainable depends in large measure on the degree to which generalization of hapten structure is valid. This subject will be discussed later. For the moment, let us consider a typical fractionation experiment in which crude lipids, whose water-soluble contaminants have been removed, are subjected to fractionation on silicic acid by gradient elution, the individual fractions then being assayed with antiserum by complement-fixation. Several experiments of this type have been described (18, 19). What will generally be observed is that immunological activity is associated with two groups of fractions, namely, those eluted rapidly with the neutral lipids, and those eluted more slowly with the amino-phosphatides. The first group will be found to be strongly anticomplementary, whereas the second group will not. If, before making the aqueous suspension of these fractions, some lecithin (in an organic solvent) is added to each, the fractions of the first group will usually lose their activity, whereas activity will be retained by those of the second group. This procedure is not entirely satisfactory for two reasons. It does not fractionate the lipids in any systematic way, and the quantity of lecithin added bears no constant relation to the quantity of material in the test fraction. Both of these undesirable features may be readily eliminated. In the first place, by using a discontinuous gradient elution scheme, a smaller number of lipid fractions may be collected; these will show the properties of the known lipids constituting the bulk of the material under study. The final fraction, eluted with methanol, will probably be inactive and consist of choline-containing phosphatides. This fraction may be used as the source of lecithin. Now if all fractions are made up to the same volume, then by combining equal volumes of any given fraction with the last fraction (choline-containing phosphatides), the amount of lecithin added will be in approximately the same proportion to the active lipid as it is in the original crude lipid mixture. We have thus available a systematic method of examining the total lipid extract of an organ or tissue for its hapten content with respect to the analytical reagent,

an antiorgan serum. The number of active substances found will depend on three factors: the specificity of the antiserum, and the number and relative concentrations of haptens in the original lipid extract. This procedure has recently led to the confirmation of the presence of a novel lipid hapten in human tissues,[4] the existence of which was indicated (20) in an earlier study of antihuman tumor serum specificity.

STRUCTURE OF LIPID HAPTENS

It seems reasonable to predict that the adsorption affinity of lipids for silicic acid will prove to be a more substantial criterion of lipid structure than solubility properties, which are so frequently employed even today. When it was found that a rat lymphosarcoma hapten was eluted from silicic acid just after the main aminophosphatide fraction (17), it was assumed that this hapten would prove to be a phosphatide (18). This thinking was influenced considerably by the fact that cardiolipin, the only pure animal lipid hapten known, was a phosphatide. Cytolipin H activity was eluted just before the main amino-phosphatide fraction (19), a position consistent with that expected for a phosphatidic acid like cardiolipin. The active fraction as eluted from the column contained considerable phosphorus, but the pure substance was subsequently found to be free of phosphorus. This observation was influential in reorienting our thinking with regard to the structure of lipid haptens, despite the suggestive evidence based on adsorption affinity for silicic acid.

Let us now consider the structures of the two pure haptens so far obtained from animal tissues. On the basis of analyses of the sodium salt of cardiolipin for carbon, phosphorus, fatty acids, glycerol, and sodium and the identification of a polyglycerol ester of phosphoric acid among the hydrolytic products, Pangborn (21) suggested the structure of cardiolipin to be glyceryl-phosphoryl-glyceryl-phosphoryl-glyceryl-phosphoryl-glycerol, in which each of the six available hydroxyl groups on the four glyceryl residues was esterified with an unsaturated fatty acid, five with linoleic acid, and one with oleic acid. Very recent evidence based on oxidative degradation of deacylated polyglycerophosphate (22) and paper chromatographic methods (23) suggests the correct structure of the molecule to be simpler, namely, glyceryl-phosphoryl-glyceryl-phosphoryl-glycerol, in which each of the four available hydroxyl groups on the two terminal glyceryl residues is esterified with fatty acid, and a single hydroxyl group on the central glyceryl residue is free (Fig. 1). Problems in the preparation of this material and its use in serodiagnosis of syphilis have been reviewed with great clarity by Pangborn (25, 26, 27). She has emphasized the effects of lipid-lipid interactions and noted that the quantity of cardiolipin in the optimally reacting mixture is small in proportion to the quantity of auxiliary lipid, and further, that specificity as well as sensitivity depend on the proportions of the three components (cardiolipin, lecithin, cholesterol) (25). Pangborn utilized data obtained by both complement-fixation and flocculation methods for estimating the reaction of cardiolipin with human antibody. Studies in recent years have extended such observations to rabbit antibody and the quantitative precipitation method (13). The value of lecithin apart from that of overcoming anticomplementary activity was noted (13). It has also been found (28) that antibody reacting with cardiolipin will react (fix complement) with relatively large amounts of lecithin, although substantial differences in reactivity were recorded among three lecithins (two synthetic, one naturally occurring) tested with the same antiserum. It was not suggested that this represents a true cross reaction. Such observations emphasize another limitation in the study of lipid specificity, namely, that, compared with water-soluble substances, specific reactions must be judged over a narrower range of concentrations. This is so because we are not yet able to determine what quantities of various lipids react nonspecifically with complement components, reactions that may occur because one of these components is a lipoprotein.

Little information is as yet available on the extent to which structural analogues of the cardiolipin molecule react with anticardiolipin. On the basis of the revised (diphosphatidyl glycerol) structure, the glyceryl phosphatide models prepared by Baer and Buchnea (29) assume increasing relevance and their activity is not unexpected. It may be pointed out that the new cardiolipin structure, in contrast to the former one, offers a large segment of polar residues (phosphate-glycerol-phosphate) unhindered by hydrocarbon chains, and this feature may be of considerable aid in eventually accounting for the specificity of cardiolipin reactions.

After it had been isolated in a pure state, cytolipin H was found to be composed of four molecular residues: fatty acid, a long-chain base, glucose, and galactose in equimolar proportions (1, 2). Since then the fatty acid residues have been established by paper chromatography and gas-liquid partition chromatog-

[4] L. Graf, M. M. Rapport, and R. B. Brandt, unpublished studies.

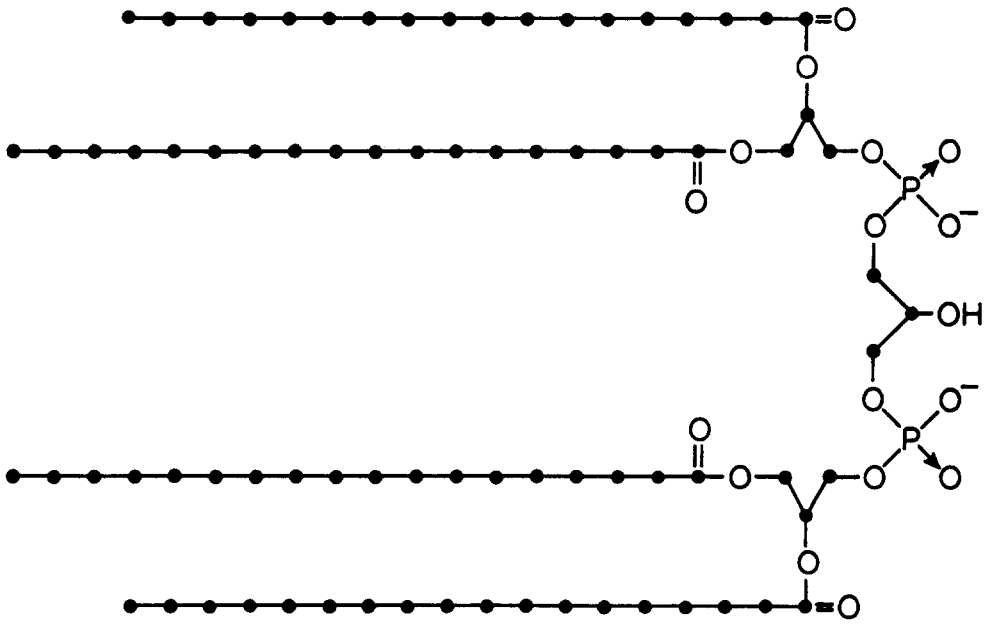

FIG. 1. The structure of cardiolipin (22, 23, 24).

raphy to be principally a combination of lignoceric, behenic, and palmitic acids (30); the long-chain base has been found to be sphingosine (30) by the decisive method introduced by Sweeley and Moscatelli (31); and the carbohydrate configuration has been established to be that of the disaccharide lactose using the sensitive immunochemical method of hapten inhibition (32). Structurally, cytolipin H is a lactoside of ceramide (Fig. 2), and it is therefore similar to cerebrosides in having a ceramide aglycon, but differs in being a glycoside of a disaccharide rather than a monosaccharide. This type of structure provides a simple solution to the problem of how a high degree of immunological specificity can be associated with lipids, which are relatively small molecules whose pattern normally permits only a limited number of structural variations. The combination of lipid residues with oligosaccharides permits a large number of possible variations to be incorporated into a class of small molecules which have the capacity to achieve high particle weights in an aqueous environment without the formation of covalent linkages.

Before considering what special assets accrue to this unique structural design and what advantages may be taken of this, let us first ascertain whether either of the two pure hapten models, phosphatide or glycosphingolipid, offers indications for the generalization of chemical structures with haptenic properties.

OTHER PROBABLE SPHINGOLIPID HAPTENS OF ANIMAL TISSUES

In their last communication on the "heterogenetic hapten" of horse kidney, Landsteiner and Levene (33) reported that the purest material they had obtained was free of phosphorus and that carbohydrate was present among the hydrolysis products. Similar results were recorded by Brunius (34). A lipid hapten from bovine brain was reported by Schwab (35) to be a "protagon" fraction, i.e., an ether-insoluble fraction in

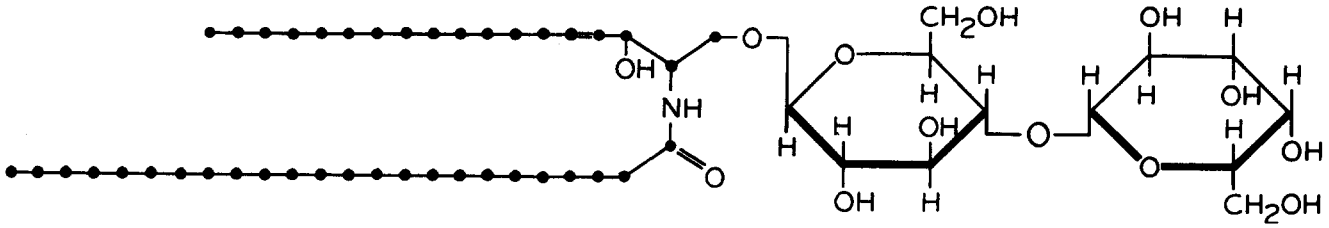

FIG. 2. The structure of cytolipin H (2, 30, 32).

which sphingolipids (usually sphingomyelin and cerebroside) are concentrated. The haptenic activity of a lipid isolated from bovine spleen, lung, and liver, and thought to be "polydiaminophosphatide" (36), a chemical structure that is no longer valid (37), was clearly associated with a sphingolipid fraction. Much of the effort directed toward isolation of blood group substances from erythrocytes of both horse and man has yielded glycosphingolipid fractions (38, 39), and most recently, a sheep erythrocyte lipid with Forssman hapten activity was found to belong to this group of compounds, since it contained both carbohydrate and lipid base residues (40). With the exception of the last substance, there is little to be gained by detailed discussion of these results since pure materials were not obtained. The sheep erythrocyte hapten, however, was reported to be pure and will be discussed below.

No comparable list implicating phosphatides as lipid haptens of animal cells can be assembled at present. Admittedly, the list of sphingolipids is weighted, since careful reading of the papers indicates some of these fractions to be water-soluble; and, according to our operational definition, such substances might not be classified as lipids since they would be expected to appear in the methanol-water layer rather than the chloroform layer after washing the chloroform-methanol extract. This would be particularly true of erythrocyte glycosphingolipids as the number of monosaccharide residues increased. In any case, it is apparent that there is a high probability that glycosphingolipids are a general model for animal cell haptens. If one attaches carbohydrate residues to lipid residues, can the resultant molecule still be classified as lipid? At what point does glycolipid become lipopolysaccharide? This question cannot be easily answered. Aside from indicating the procedures that one employs to isolate, characterize, and study the chemical and physical properties of a substance, the question has little meaning. In the case of cytolipin H, it is clear that chemically the substance behaves as a lipid, whereas immunologically it reacts principally as a carbohydrate. What is most suggestive about this design of at least some cellular haptens as glycosphingolipids is related to the finding that sphingolipids such as sphingomyelin and cerebroside are almost certainly myelin components, and therefore presumably components of membranes. In ceramide we have two lipid residues by means of which an oligosaccharide label can be cemented into the lipophilic portion of a cell membrane. This label, which is now part of a high particle weight structure by virtue of the mass of the cell, may then exercise its influence in a variety of reactions, which are most familiar to the immunologist because of the existence of antibodies which produce agglutination, complement-fixation, and cytotoxicity. Much of the serological work on individual cell labels has been restricted to erythrocytes because of their obvious technical advantages (discrete cells, self-contained pigment). These cells have proved to be an unsatisfactory source of material for most chemical studies for reasons which are both known (difficulty in removing hemoglobin) and suspected (low concentration of individual hapten; presence of many haptens). Studies by several groups of investigators (41 to 45) suggest the presence in erythrocytes of many substances that may have haptenic function. It is to be hoped that cells grown in culture will offer a far simpler source of material for chemical study in much the same way as have tumor tissues.

If most lipid haptens should be glycosphingolipids, there would be a simplification in their isolation and identification. As a result of very recent studies (17)[4,5] a number of lipid haptens (from rat lymphosarcoma, horse kidney, human colon carcinoma) were found to be eluted from silicic acid together with aminophosphatides. Because of present analytical limitations, the further fractionation of phosphatides in this material would be exceedingly difficult. However, the separation of a sphingolipid from the phosphatides is relatively straightforward, and when this was done with Forssman hapten from horse kidney,[5] the active fraction was indeed found to be associated with carbohydrate-containing lipid. Purification can thus be based on carbohydrate content, and should specific monosaccharide residues be identified, an even more decisive criterion would be available. Furthermore, it is possible to gain some valuable information on the number of monosaccharide residues present in the molecule from the solvent required to elute the lipid from silicic acid, assuming no charged chemical groupings are present, such as sulfate, phosphate, or carboxyl (sialic acid). Discrete separations can be made between sphingolipids containing one and two monosaccharide residues, and between the latter and the main ninhydrin-positive phosphatide fraction (46).[6] The three haptens listed above, if they are glycosphingolipids, should contain only three, or possibly four, monosaccharide residues. This degree of molecular complexity is capable of exact solution with relatively small quantities of material. The main obstacles to be overcome are the poor criteria of purity and the shortage of techniques

[5] M. M. Rapport, L. Graf, and N. F. Alonzo, unpublished observations.

[6] M. M. Rapport and N. F. Alonzo, unpublished observations.

for purification, the latter resulting principally from the limited solubilities of these molecules. This view may be optimistic because it leans heavily on experience associated with the isolation of cytolipin H, whose structure, in retrospect, appears to be the simplest prototype. The properties of this substance do not differ appreciably from those of cerebrosides, so that a very considerable background of chemical experience is applicable to this hapten. The only serious loss in applying to cytolipin H methods useful for characterization of cerebrosides was the failure to gain useful information from infrared spectra. One of the intriguing aspects in the study of more complex lipid haptens is how chemical properties will be modified as additional monosaccharide residues are added. With the availability of synthetic methods (47) it may not be necessary to await isolation of natural products for complete answers, although, at the moment, the approach based on identification of natural products still offers an advantage in efficiency and considerably more from the standpoint of possible application.

Exact knowledge of the structure of lipid haptens, which is possible because of the combination of small size and basic simplicity of design, has an immediate consequence in indicating methods for producing specific antibody which circumvent the haptenic limitation. Antibody specific for a carbohydrate determinant can be prepared by immunization with protein chemically coupled to an oligosaccharide through an azophenyl linkage (48). Thus antisera prepared against bovine -γ- globulin-azophenyl lactose, or porcine -γ- globulin azophenyl lactose, that react with the lactose determinant as shown by precipitation with egg albumin-azophenyl lactose, also react with cytolipin H.[7] Studies are in progress which have as their objective to compare the avidity for cytolipin H of antibody directed against a lactose determinant and of antibody prepared against tissue fractions containing cytolipin H. If the avidity of the latter is found to be greater, as preliminary results suggest,[7] it would indicate that antibody formed against cytolipin H is complementary to part of the lipid structure (possibly the polar groupings of sphingosine) as well as the carbohydrate. With haptens containing three monosaccharide residues it might be expected that the proportional contribution of the lipid would be measurably less, and that a higher degree of specific reaction with antibody would be observed. Increasing the number of carbohydrate residues should also affect antigenic strength, as expressed in the capacity of a mixture of haptens associated with protein (either in tissue fractions or in "combination immunization" techniques) to elicit antibody reacting with individual components. The phenomenon of antigenic competition among haptens, sometimes shrouded with the nebulous concept of "availability," is indicated in experiments in which cytolipin H was found to be a poor competitor in the presence of a rat tissue hapten of greater chemical complexity.[1]

[7] L. Graf, J. Yariv, and M. M. Rapport, unpublished observations.

SPECIFICITY OF LIPID HAPTENS

The specificity to be considered here is one of distribution: Are these relatively low molecular weight lipid haptens found only in specific cells, and if so, are they present in corresponding cells of different animal species? If the molecular residues composing these haptens are part of the ordinary pool of structural components and the hapten pattern is so simple, how many variations occur and how often is the same pattern used? The answer to these questions is of paramount importance for our understanding of the biological significance of lipid haptens or appreciation of how they may be used for some practical purpose. Answers are not readily deduced from available evidence because of inherent defects in the methods employed to obtain the data. For example, extensive surveys have so far been limited to Forssman haptens and blood group haptens (49, 50, 51) (cardiolipin is distributed almost ubiquitously). Neither of these two types of hapten activity represents a discrete chemical entity. Both human A and B group specific substances are present in red cells and tissues as either alcohol-soluble substances (presumably lipid haptens) or as water-soluble peptide-polysaccharide combinations (50, 51). Forssman haptens are defined as substances which react with antibody to sheep erythrocytes; if the antigen mosaic of these cells is complex, then many different molecules, either identical with one of the erythrocyte antigens or capable of cross-reacting with antibody to them, will be included in any measurement of "Forssman hapten." Furthermore, antisheep erythrocyte sera may vary qualitatively and quantitatively in antibody content. It is small wonder, therefore, that the distribution of Forssman hapten has been found too broad to be instructive. Nevertheless, it is clear that studies of the Forssman system established that lipid haptens have the property of crossing species lines, and, as a consequence, the terms "heterogenetic" and "heterophile" were introduced. Although these terms are sometimes used synonymously with "Forssman," they have a broader meaning since they

are applicable whenever haptenic activity in one species is observed with an antibody system "homologous" to a different one. However, this terminology is so ambiguous that organ specificity (in the sense of reactions common to a tissue such as brain or heart, regardless of the animal of origin) is not referred to in the literature as "heterophile" (52). Such terms reflect the inability of serological methods per se to supply definitive answers. Chemical methods may eliminate much of the confusion, but a combination of chemical and serological analyses will doubtlessly be required to secure stable evidence. It is worth mentioning that the distribution of individual lipid haptens may not be as broad as studies of the Forssman model indicate. The rat lymphosarcoma hapten system, for example, showed no cross reactions with lipid extracts from tissues of five heterologous mammalian species (53).

Limitations of serological methods are attributable to imperfect specificity of antisera and unreliability of quantification. Their strength lies in sensitivity. Chemical methods, on the other hand, offer reliable quantification but suffer from lack of sensitivity, and also from lack of specificity although the range of imperfection is narrow. The two methods can complement each other nicely if the limitations are kept in mind. A combined serological-chemical approach to analysis of lipid hapten distribution has been started with the cytolipin H system using one of the more specific antihuman tumor sera.[3]

Before discussing some of the early results, let us first look at an interesting facet of chemical investigation that is a direct consequence of isolation and structural identification of cytolipin H. A molecule with similar composition had been isolated from bovine spleen by Klenk and Rennkamp in 1942 (54) as a by-product of the study of gangliosides, and it was quickly found[1] that lipid extracts of this organ did indeed react with antisera that gave reactions with cytolipin H. Rigorous confirmation by chemical and serological comparison of the substances was required, and therefore several pure preparations of an active glycolipid were isolated from bovine spleen. These preparations and cytolipin H gave very similar values on chemical analysis, and small differences in rotation and unsaturation that were found could be attributed to variations in the lipid residues (55). Although more than 90% of the lipid base was sphingosine and the fatty acids were predominantly lignoceric (C_{24}), behenic (C_{22}), and palmitic (C_{16}) acids, the proportions varied from one preparation to the next, as did the other fatty acid residues. Serologically the material from bovine spleen was indistinguishable from that isolated from human tumor (55). An earlier report became especially interesting now, namely, that a pure sheep erythrocyte hapten had been found to contain fatty acid, a lipid base, galactose, and galactosamine (40), and it therefore seemed probable that an increasingly large number of different glycosphingolipids with two monosaccharide residues might be found as isolation and chemical identification of lipid haptens progressed. For this reason the generic term "cytoside" was introduced (56) to simplify discussion of these different molecules and their immunological individuality. The term cytoside to describe a glycosphingolipid with two monosaccharide residues is similar to the term cerebroside, which is commonly used to describe a glycosphingolipid with one monosaccharide residue. Introduction of such a term has additional validity on the basis of chemical classification based on adsorption affinity for silicic acid. As a result of studies of an appreciable number of human tissues and a limited number of animal tissues, it is apparent that cytosides are much more rarely encountered than had been surmised.[3] Furthermore, the description of the sheep erythrocyte hapten as a cytoside may be questioned for the following reason. Cytolipin H and bovine spleen cytolipin are similar to cerebrosides in their solubility characteristics. In particular, they are completely insoluble in water. The sheep erythrocyte hapten, in contrast, is reported to be water-soluble (40), and it is difficult to reconcile the small differences in chemical composition with such a profound difference in physical properties unless the chemical structures differ greatly. Using a combination of chemical and serological analyses, both sheep and dog spleens were found to contain small quantities of cytoside having the serological reactivity of cytolipin H.[3] However, rodent (rabbit, rat, mouse), cat, hog, and horse spleens were found to be devoid of cytolipin activity and to show no indication of the presence of other cytosides.[3] The findings suggest a slight revision in terminology. The H in cytolipin H, originally introduced to indicate "human," should therefore be considered to represent "heterophile" or "heterogenetic."

Among human tissues, examination of a limited number of specimens showed cytolipin H to be absent from brain, heart, kidney, and a number of tumors.[3] This hapten was found also to be one of the glycosphingolipids of pooled horse erythrocytes but not of bovine or human erythrocytes (43). These results suggest that lipid haptens are associated with particular cells, that a given structural pattern may be repeated in different species, and that distribution may be much more restricted than earlier studies of Forssman

hapten led us to believe. Obviously much more work will be required before we can formulate valid generalizations.

LIPID HAPTENS OF HUMAN TISSUES

How many different lipid haptens are found in cells of a single species? Extensive studies are available only for those of human origin. We can be reasonably certain of seven: cytolipin H, cardiolipin, the hapten in tumors of the gastrointestinal tract previously referred to, a Forssman hapten, the alcohol soluble group A and group B substances of erythrocytes, vascular intima, islets of Langerhans, gastric parietal cells, and lung (50, 51), and the Rh hapten (57, 58). Five of these must still be isolated, characterized, and identified. There are, in addition, at least two other lipid haptens that appear to be organ specific (in the sense that similar tissue from a different species of animal contains significant quantities), namely, one from myocardial sarcoplasm that can be distinguished from cardiolipin (59, 60), and one from brain (61, 62). It is probable that these nine substances will be serologically unrelated, but studies directed toward answering this question have not yet been reported. Other potential haptens exist wherever carbohydrate residues are found bound to lipids, such as the cytosidelike fraction found in low concentration in human kidney (that does not show cytolipin H activity).[3] It is not, therefore, an unwarranted assumption that many individual cell types may have distinguishing hapten labels in their membranes by means of which serological identification can eventually be made. The number of lipid haptens may be increased greatly by including those not present in man, some of which are indicated in an earlier review of heterophile reactions (63). The fact that many of these substances are found in different animals offers a remarkable opportunity for overcoming the greatest obstacle in isolation work—finding an adequate source. This fortunate state of affairs actually led to the isolation and identification of the cytolipin molecule seventeen years before its haptenic function was established.

THE ROLE OF LIPID RESIDUES IN LIPID HAPTENS

As has already been indicated, the part played by the lipid residues in lipid haptens is poorly understood. From a theoretical standpoint, these residues may well serve as points of attachment of the haptens to organized segments of cellular structure such as membranes. There may therefore be no well-defined lipoprotein molecule of which they form a part and which was suggested by evidence based on complete antigenicity of cell fractions (64). Other, still preliminary, evidence implicates the participation of a portion of the lipid residue in the fragment of structure to which the antibody site is complementary.

From a practical standpoint, the presence of lipid residues in lipid haptens requires a distinctive approach in correlating structure with activity, and even for tracing activity during purification procedures. Lipids apparently display their specific reactions with proteins far more efficiently in concert with other ("auxiliary") lipids than alone. The effect of lipid-lipid interactions may be interpreted only in part on the basis of increased dispersion in an aqueous medium. The effect of cholesterol, a more hydrophobic substance than the hapten itself, and a common constituent of membranes, cannot be explained in this way. The requirement for auxiliary lipids is even seen in lipid-protein interactions involving lipids that disperse readily in water (12, 33, 34). Although only superficial information is available, there are indications that auxiliary lipids (lecithin, cholesterol, sphingomyelin, cerebroside), despite their ubiquitous distribution, may specifically activate different haptens. In part, the auxiliary lipid requirement for immunological activity is readily understandable on the basis of converting a monovalent molecule into a polyvalent aggregate, which can then react with antibody to form an immune complex with properties similar to those formed by water-soluble polyvalent antigens and antibodies. What is loosely thought of as "dispersion" may, in fact, be the formation of mixed micelles with optimally reactive molecular arrangements.

In studies with substances containing hydrophobic chains, it should also be appreciated that a considerable driving force favoring reaction may be associated with decreased water-solubility (65). It might thus be expected that the inhibition of lipid hapten-antibody reactions by water-soluble oligosaccharides would be less efficient than inhibition of polysaccharide-antibody reactions.

PSEUDO ANTIGENS AND OTHER LIPIDS

This review will close on a disagreeable note, namely, an attempt to separate true immunological reactions from studies which appear in some vague way to be relevant, but are not. Such studies provoke numerous questions on whether a relation exists among them because they all involve lipids associated with the

problem of cancer. Thus within a rather short period of time, in addition to cytolipin H, malignolipin (66), carcinolipin (67), a "crystalline antigenic phospholipid" (68), and "lipid antigens" useful in serodiagnosis (69, 70, 71) have been described. These appear to be unrelated. Cytolipin H has been discussed at length in this review. It is not "cancer specific," despite inadvertent misrepresentations to this effect (7). Malignolipin is presumably a phospholipid; although isolation of the pure substance was reported, adequate physical and chemical characterization was not presented. It has not been found to have antigenic or haptenic properties. Carcinolipin is the name assigned to a lipid of undefined structure that was detected originally in egg yolk, because of its enhancing effect on protein biosynthesis. It is present in rat tissues and is elevated in tissues of tumor-bearing animals. No immunological properties have been associated with this material. The so-called "antigen" of the Penn test (69), and a similar phenomenon described by Eisenstaedt (70, 71), is not an antigen. This terminology is used because the technique of the test bears a resemblance to seroflocculation methods that have been used with some lipid haptens. While the physicochemical basis of the reaction is not well understood, it appears to involve an effect of serum proteins in disturbing a metastable lipid sol. The protein composition of cancer sera may be such as will cause the sol to flocculate. Certain proteins appear to be more effective than others (72), and several lipids may be used to prepare the sol (73). The use of the term antigen here is indeed unfortunate. Finally a "crystalline antigenic phospholipid" is reported to have been isolated from human cancer tissue (68). This study does not appear well grounded and further consideration must await independent confirmation of the findings. It is essential to keep these different studies well separated in order to avoid prejudging the merits of any one on the basis of the success or failure of the others. Aside from the value that new substances offer in terms of potential utility, much more substantial gain may attend attempts to place them in proper perspective by chemical and physical characterization. It is essential to establish the nature of the reactions in which these lipids participate, if we are to expect any rational exploitation of their specific properties.

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