Immunologic Studies of Autochthonous Cancer
An Evaluation of Several Procedures*

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INTRODUCTION

The unique and complex problems in studies of spontaneous tumors of animals derive from the fact that the limiting frame of reference comprises a host response to an invasive, replicating cell presumably of autologous origin. Unlike the situation in many infectious diseases, immunologic phenomena attributable to reactants of exogenous origin are not considered admissible in studies of autochthonous cancer. As in the area of auto-antibody production, this limiting condition imposes rigorous requirements for the immunologist. Evidence for an immune response to spontaneous tumors must be buttressed by the demonstration of antigenic activity which clearly differentiates the neoplasm from normal host tissues.

The recurrent reports and disavowals of specific antibody production to autologous cancer tissues have led to the consideration of several hypotheses. They are listed, not in the sense of being all-inclusive, and without further comment as to their validity, since a critical evaluation has not yet been undertaken and the probability exists that a single mechanism may not be equally tenable for all situations.

1. Tumor cell multiplication progresses in the absence of antibody production.
   a) The genetic changes which characterize neoplastic cells are not reflected in antigenic differences, morphologic and functional disparities notwithstanding.
   b) Tumor cells are deficient in antigenic components present in normal tissues.
   c) Tumor cells proliferate only when a state resembling acquired immune tolerance or immunologic paralysis intervenes.

2. Tumor-specific antibody is produced but is not amenable to extensive in vitro investigation, because it is of the cell-dependent type, which mediates delayed hypersensitivity reactions, or of the reaginic, skin-sensitizing variety.

3. Circulating tumor-specific antibody is synthesized but is not uniformly detectable in the serum because it is bound to the cells which incite its production.

4. Antibody is synthesized by the tumor cells and is directed against host tissue components.

5. Circulating tumor-specific antibody is produced which reacts extensively with a variety of normal tissue components.

The remainder of this report will be based on the assumption that circulating antibody, endowed with varying degrees of tumor specificity, is present in sera of humans or animals bearing spontaneous tumors. Several experimental applications to the problem will be discussed primarily in terms of the interpretational restrictions imposed by the methodology.

A. COMPLEMENT FIXATION IN ANTIGEN CHARACTERIZATION STUDIES

1. THE FIVE-UNIT METHOD IN STUDIES OF TUMOR-SPECIFIC ANTIGENS

The complement (C') fixation reaction has been used extensively for the immunologic characterization of a tissue lipide with potential tumor specificity (12, 44). It may therefore be appropriate to consider the applicability of current C' fixation procedures to this problem. The investigator who contemplates this approach faces the choice between one of two general types of C' fixation techniques: the conventional method which utilizes five 50 per cent units of C' (C'H50) such as described in (38) and the so-called quantitative technic with 50
or more C'H₅₀. Certain more general aspects of the two procedures have been evaluated previously (20, 33). For the present purposes, it may be appropriate to discuss other features with specific applicational reference to the estimation and characterization of lipide-soluble tissue extracts from normal or cancerous tissues.

The technic used by Rapport and Graf in their studies of tumor lipides is essentially that developed by Wadsworth, Maltaner, and Maltaner (54) and also studied extensively by Rice (47, 48). The results are expressed in terms of the quantity of antigen or antibody required to yield 50 per cent hemolysis in a C' fixation reaction with 6 C'H₅₀, and a constant amount of antiserum or antigen in the presence of varying quantities of the other.

The limitations of this method in studies of antigen characterization may be summarized as follows.

When 6 units of C' are used in the initial fixation reaction, the subsequent addition of sensitized erythrocytes to the antigen-antibody-C' mixture, for the lytic estimations, can lead to one of three results and implications, assuming that all the controls are in good order.

a) No lysis.—This finding means that at least 5.5 of the initial 6 C'H₅₀ were consumed in reacting with the antigen, the antibody, or the specific aggregate, since 0.5 C'H₅₀ produces less than 10 per cent hemolysis.

b) Partial lysis.—The number of C'H₅₀ fixed in the reaction may be localized between the limits of 4.5 to 5.5 units, since the range of partial lysis requires 0.5 to 1.5 C'H₅₀.

c) Complete lysis.—Less than 4.5 C'H₅₀ were fixed.

It is thus readily apparent that the use of 6 C'H₅₀ leads to a quantitative estimate only within a narrow range of C' action—namely, one C'H₅₀. The remainder of the C' serves to swamp various anticomplementary effects which may be very troublesome in tests with less than 5 or 6 C'H₅₀. A consequence of this severe restriction in the range of C' action is that heavy reliance must necessarily be placed upon the differences in test results with the twofold dilution sequences of antigen or antibody. It follows, then, that an antigenic variable which is expressed by less than a 50 or 100 per cent difference could be attributable to "experimental error," which is of the same order of magnitude in these reactions.

A simple illustration may clarify this point. The identification by Pangborn of cardiolipin as the major antigenic constituent in crude lipide extracts containing the Wassermann antigen (39, 40) led to the isolation of a similar phospholipide from wheat germ, called sitolipin (52). Comparative serum titer estimations with these two phospholipides by conventional complement fixation and flocculation procedures supported the notion that they were identical (46). When quantitative C' fixation technics with 50 C'H₅₀ were applied to this problem it was readily discernible that the phospholipide of plant origin was indeed similar but was not identical to those extracted from mammalian tissues. Sitolipin proved less efficient than the beef or human heart cardiolipins in precipitating the Wassermann antibody or in C' fixation studies with this antibody as derived from a variety of sources including sera of human syphilisics (36, 37).

The data in Chart 1, which were obtained with the 6-unit method, describe the C' fixation results in the three different zones of antigen-antibody interaction. The vertical line essentially parallel to the ordinate roughly describes the region of antibody excess, becoming curved at equivalence zone ratios and asymptotic to the abscissa in the region of antigen excess. At antigen excess ratios, a divergence may occur in that some immune systems exhibit more pronounced inhibition with excess antigen (Type II) than do others (Type I). It is apparent from an inspection of these curves that major segments of each type of curve contribute

1 A. G. Osier, J. H. Strauss, and B. Lowenstein, unpublished observations.
little to the characterization of the antigen-antibody reaction under study. With a Type I curve, marked variations in antigen or antibody concentrations produce no detectable change in the C' fixation pattern. It is only in the restricted curvilinear portion of this curve that alteration in the amount of either antigen or antibody will seriously affect the outcome of the reaction. These considerations may account for the wide variations encountered in those experiments which "did not permit the quantity (of antigen) to be assessed accurately" despite "corrections for the fixability of C' in terms of the deviation of the standard antigen from its assigned value" (45). In the case of Type II curves, the region of antigen excess depicted by the right-hand linear portion can be applied to problems of antigen estimation. Unfortunately, the Type II pattern does not describe all immune systems, and the factors which lead to either of these two reaction patterns are not entirely clear.

The inference to be drawn from these comments is that this C' fixation system provides a relatively insensitive instrument for differentiating variations in antiserum or antigen activity when the increments are less than 50 per cent. As noted in references (20) and (33), these difficulties emerge from the design of this type of C' fixation procedure which utilizes a low level of C' and thereby circumscribes the limits within which only relatively narrow ranges of antigen and antibody interaction can be studied. The data shown in Chart 2, which reproduce a portion of those in Figure 14 of reference (44), corroborate this statement. It may be seen that, as the initial amount of C' is increased from 4.5 to 24 C'H₉₀, each curve exhibits greater discriminatory potential in terms of the range of antigen or antibody concentrations which alter the test result.

Some of the advantages which accrue from the use of a relative excess of C' such as 24, 50, or 100 C'H₀ in C' fixation studies designed to elucidate certain aspects of antigen-antibody interaction in highly dilute systems have been discussed previously (33). The following section of the article will therefore consider additional applications particularly as they may be referable to immunologic characterization of tissue antigens.

2. THE QUANTITATIVE METHOD
   a) Studies with lipide antigens.—One problem of crucial importance with respect to lipide-soluble antigens needs restatement. It has been amply demonstrated in (26) that the specific antigenic activity of these substances varies dramatically with the presence and concentration of auxiliary lipides, themselves lacking reactivity with antibody. Cardiolipin in aqueous solution can combine with Wassermann antibody but does not fix C' in this interaction unless cholesterol is added. The cholesterol is considered to facilitate aggregation by providing a particulate surface for the adsorption of cardiolipin, whose unit molecular weight approximates 2000 or less. The tendency for cholesterol crystals to aggregate spontaneously in isotonic saline must also be controlled with judicious addition of lecithin in carefully calibrated quantities. This cardiolipin-cholesterol-lecithin complex may then be used in studies of antigenic activity based on aggregation with antibody. Cardiolipin alone will combine with its specific antibody and act as a hapten inhibitor. When new lipides with antigenic activity like cytoplipin H are brought to investigation, estimations of tumor specificity can be subject to meaningful interpretation only when each tissue extract is carefully standardized with respect to the requirements for adjunct lipides. This requirement renders studies of this type increasingly tedious and complex, and even when fulfilled may be applicable only to lecithin and cholesterol. The use of newer chromatographic methods for lipide separation should be helpful in providing more highly purified products so that the influence of lipide-lipide interaction on antigenic behavior may be further clarified. The experiments with cardiolipin now to be outlined may serve as one example of the applicability of C' fixation analyses to problems of organ and species specificity. Two technical problems required resolution. The first dealt with a supply of purified cardiolipin preparations which were extracted from cardiac tissue of bovine and human origin, and from human liver.
according to the method of Pangborn (40). The activity of these preparations with lecithin and cholesterol was determined in preliminary trials and preceded the type of experiment such as described in Chart 3 and in (37). It may be noted from these data that the concentration of Wassermann antibody is expressed in weight units. The procedure for obtaining these values with lipide antigens was developed for this study and provided an independent means for confirming the results of the C' fixation experiments. As indicated in the chart, two sets of reactions were set up, one with varying levels of beef heart cardiolipin, the other with the human preparation, both reacting with rabbit antibody to the beef heart phospholipide. The antibody N levels obtained with this serum were 27.4 μg. for the homologous bovine preparation and 28.3 for the human heart cardiolipin. The C' studies were carried out at 37° C., with 1 ml. of a serum dilution calculated to yield 0.55 μg. of antibody N on the 27.4 μg. basis. Except for the appropriate controls, each tube in the C' fixation experiment contained the same level of antibody and 50 C'H₅₀, an amount chosen so as to leave a considerable excess. Of interest in this experiment are the identical values obtained with the two cardiolipins in the rising portion of the curves comprising the antibody excess and equivalence zone regions. A slight disparity, never exceeding more than 3 C'H₅₀, is apparent with increasing quantities of antigen, attaining peak values of 19 and 17 for the beef and human cardiolipins, respectively. These differences barely exceed the experimental error. With some sera, a reversal in rank was noted. This experiment, like others with many different immune systems, was carried out with a range of antigen concentrations that varied from 0.07 to 11.0 μg. In view of the relative excess of C', all regions of the antigen-antibody reaction were available for comparative studies.

It became clear from these studies that beef heart, human heart, and human liver cardiolipins were immunohemochemically indistinguishable, thus providing a rational basis for the so-called non-specific Wassermann test. On the assumption that the Wassermann antibody represents an immune response to tissue rather than to Treponema pallidum, it is clear that beef heart may therefore serve as an excellent and more readily available substitute for the human tissues in routine tests.

b) Antigen estimation by means of C' fixation.—The data in Chart 3 also provide a sensitive and specific basis for estimating the cardiolipin content of different tissues, normal and neoplastic. As may be noted, microgram fractions of cardiolipin can be assayed by means of a reference serum, with an accuracy of about 10 per cent. Further, some of the difficulties encountered in studies of lipide antigens may be more amenable to resolution with an antigen assay system of this type through the utilization of inhibition studies with those lipides that fail to aggregate in the absence of cholesterol. As applied to cardiolipin, crude lipide extracts of various tissues freed of cholesterol can be assayed for this phospholipide in terms of inhibition of C' fixation with the cardiolipin-cholesterol-lecithin complex.

Levine and his colleagues have described extensive applications of the quantitative C' fixation method to the problem of antigen estimation and characterization. Among these may be mentioned the human low density lipoproteins (23), a heat-labile α-2-glycoprotein (48), and the comparative antigenic activities of proteins and deoxyribonucleic acid extracts obtained from ruptured T4 bacteriophage (4, 22, 24, 31). The enhanced C'-fixing activity of thermally denatured DNA as compared with that in the unheated nucleic acid is also described. More recent studies with a micromethod have also been developed recently. An interesting application of the quantitative method in delineating those portions of a protein molecule concerned with enzymatic and antigenic activity may be found in the review by Brown et al. (5). Evidence is presented in this paper that disulfide bonds play an important but quantitatively different role in the enzymatic and antigenic activity of ribonuclease. When the protein was treated with thioglycollate at pH 8.5 and iodoacetate acid was added subsequently to prevent reoxidation of the sulfhydryl residues, the derivative fixed half of the C' but possessed only 5 per cent of the initial enzy-
mic activity. The data as shown in Chart 4 (taken from that paper) allow for the further inference that the partial reactivity of the derivative cannot be due to a mixture of inert and completely active enzyme. Were this the case, the addition of this mixture in quantities greater than 0.5 μg. N would fix as much C' as did the native ribonucleases. Since this was not observed, and on the basis of chemical analyses, it was concluded that two or more disulfide bonds are essential for complete antigenic activity.

c) Quantitative estimates of the nucleoprotein-reactive γ-globulin in lupus.—In a recent, as yet unpublished study, the quantitative C' fixation procedure has been used to estimate the amount of γ-globulin in the sera of patients with systemic lupus erythematosus (S.L.E.) and other collagen diseases, capable of combining with calf thymus nucleoprotein. The essential principle involves a C' fixation assay for human γ-globulin as antigen with specific rabbit antiserum as the antibody. In effect, these determinations can be regarded as providing reproducible estimates of lupus antibody under the anomalous circumstances in which the antigens have not yet been completely identified (51).

This device is based on the well known fact that sera from patients with S.L.E. react with a variety of preparations derived from cell nuclei, as shown by Miescher and Strässle (29), Holman, Kunkel and their colleagues (7, 18), and many others. A standardized suspension of insoluble nucleoprotein particles, prepared according to (18), is reacted with an appropriate dilution of the patient's serum. In the event that the serum contains reactive γ-globulin, such as will elicit the L.E. cell phenomenon or immune fluorescence with cell nuclei, a nucleoprotein-γ-globulin complex is formed which is not disrupted by three cycles of washing and resuspension. These washings yield a final supernate containing less than 0.05 μg. of γ-globulin N. An aliquot of this nucleoprotein-γ-globulin complex dissolved in alkaline buffer is added to a predetermined quantity (3.0 μg.) of rabbit antihuman γ-globulin N. In this fashion, the γ-globulin bound to the nucleoprotein fixes C' in combination with its specific antibody in the rabbit serum. The number of C'H₂O fixed is then converted to weight units of γ-globulin on the basis of a calibration curve such as shown in Chart 5. To heighten the specificity of this reaction for human γ-globu-

![Chart 4](chart4.png)

**Chart 4.**—Fixation of C' with native RNase (△) and partially reduced RNase blocked by idioaceic acid (taken from [5]).

![Chart 5](chart5.png)

**Chart 5.**—Each symbol (▲ • ■) indicates the values obtained in one experiment with intestinal strips from a single guinea pig.

lin, the rabbit antiserum was absorbed with human α- and β-globulins, human serum albumin, and guinea pig serum proteins. The calibration curve, which is duplicated with each experiment, was prepared with a lyophilized preparation of human γ-globulin. Preliminary studies with the electrophoretically separated γ₁ and γ₂ constituents of this product showed little difference in the C' fixing activity of these two proteins with the pooled antisera used as the source of antibody.

This procedure has indicated that normal human sera possess less than 4.0 μg. of nucleoprotein-reactive γ-globulin N/ml, whereas the levels in S.L.E. generally range from 9 to 80. In individual patients, these levels vary with clinical activity, being low in remissions which occur as a consequence of steroid therapy. It has also been observed in patients with S.L.E. that serum C' levels...
vary inversely with the quantity of this γ-globulin demonstrable in the same specimen. This finding provides a basis for further exploration of the mechanism of tissue damage in S.L.E. These studies confirm others in the demonstration that the fraction of nucleoprotein-reactive γ-globulin which reacts with purified preparations of calf thymus deoxyribo nucleic acid represents only a minor portion of the total antibody response in some patients and is entirely absent in others.

C′ fixation procedures of this type may be applicable to studies of other auto-immune diseases and possibly to investigations of spontaneous cancer to the extent that the latter may be considered as an immunologic disease of autologous origin. Thus, it would be of more than academic interest to determine whether neoplastic tissues or cells contain larger quantities of bound γ-globulin than their normal counterparts, when both are obtained from the same host.

B. CYTOTOXIC ACTION OF ANTIBODY AND C′ ON TUMOR CELLS

The in vitro neutralization reaction represents another important method for studying the humoral antibody response in spontaneous cancer. For these experiments, tumor cells are suspended in a fluid reaction medium with varying dilutions of antiserum and fresh guinea pig or other serum. The effect of these additives on the viability of the tumor cells may be assessed by animal inoculation, or by a variety of in vitro procedures such as described recently by Goldberg and Green (11, 13–15) and by Winn (57, 58) among others. Roizman and Roane⁷ have recently devised an interesting method for differentiation of viable from injured or killed cells. Human epidermoid carcinoma cells (H.Ep. #2) are infected with Herpes simplex virus and then treated with fresh guinea pig serum and rabbit antiserum to the H.Ep. 2 cells. Those tissue culture cells rendered nonviable through the immune cytolytic process do not support replication of the virus and consequently fail to form plaques. The diminution of the plaque count as compared with the controls provides a quantitative estimate of the activity of the anti-H. Ep. #2 cell antibody.

Two types of problems emerge in a consideration of the many interesting studies in this direction which bear on the role of C′. The first and probably most important to the problem of cancer immunology relates to the detection and estimation of tumor-specific antigens as distinguished from normal host tissue constituents. In addition, there is the crucial question of selecting, from among the complex of antigens, the one or more which are required for tumor cell viability.

The parallelisms which may be drawn to analogous studies of bacterial and viral antigenic composition are many and obvious. A single illustrative example may be selected. The antibody response to an infection with a Type 12 hemolytic streptococcus of Lancefield’s Group A can be estimated by many methods. However, these measurements provide no insight as to the immune status of the host with respect to the specific M protein characteristic of the Type 12 organism. The amount of antibody produced to this antigen may be critical insofar as resistance to Type 12 streptococcal infection is concerned, but probably comprises only a minor segment of the total immune response to the many intra- and extracellular antigens of this organism.

It follows, then, that application of C′ fixation, hemagglutination, gel diffusion methods, etc., for studies of cancer immunology can be used for estimating the over-all immune response and in the enumeration of the number of contributory antigen-antibody systems. These procedures may also be valuable as adjuncts in the selection of the antigens which participate in the cytotoxic reactions. However, identification of the latter will undoubtedly constitute a major advance as a necessary prelude to rational efforts at immunotherapy. This information could conceivably lead to the production of specific antibody less subject, perhaps, to the complex of cross-reactions with normal tissues. In view of the potential importance of this approach, two aspects of the cytotoxic reactions in vitro may be discussed briefly.

Generally, the antisera used for these experiments are obtained from tumor-bearing hosts (9, 8, 9, 57, etc.) or following the injection of a heterologous animal (e.g., rabbit) with intact cells or their fractions (11, 13–15). The latter approach is occasionally complicated by the fact that the antibody response of individual animals which follows the injection of an antigenic mixture is highly variable with respect to any single component. This difficulty is often circumvented by absorption of the immune serum with antigens other than those under study and is based on the assumption that there is no immunologic overlap between the two. The serum titers finally obtained after these absorptions are often too low for definitive studies. Difficulties of this nature have been encountered in attempts to produce antisera specific for individual strains of Salmonella. The magnitude of
this problem may be gauged from the report of Habel et al. (16). In considering this line of approach, it should be re-emphasized that the use of antigen dilution titers to estimate the potency of an antiserum or its degree of crossreactivity does not adequately characterize the immune system under analysis unless two-dimensional titrations are also performed for comparative purposes. In studies with well characterized protein or polysaccharide antigens as well as purified poliovirus, it is usually observed that the antigen titer remains relatively constant despite wide variations in antibody levels, as may also be seen in (50, 59) (cf. also reference [4]). It has been further shown that, in the region of antigen excess, heterologous antigens may yield higher C’ fixation titers than those obtained in the homologous reaction. These cannot be interpreted in any valid fashion unless quantitative considerations are noted and applied (35).

In this light, antigen titers to one tissue preparation following absorption with seemingly unrelated crude tissue extracts of undetermined antigenic content cannot be evaluated in the absence of data pertaining to the respective serum antibody levels.

An approach which might circumvent some of these technical obstacles would rely on the capacity of tumor cell fractions to inhibit the cytotoxic effect of C’ and antibody. The combined use of in vitro measurements of cytopathic effects with animal inoculation experiments might also expedite identification of specific cancer cell antigens of transmissible tumors, essential for cell survival and multiplication.

C. IDENTIFICATION OF C’

Many in vitro studies of cytotoxicity have dealt with the role of serum C’, and some of these have been discussed in (33). The facile assumption that enhancement of the test effect by fresh guinea pig, rabbit, human, or other serum operates through the C’ system may be misleading in view of findings such as those by Amano, Inoue, and their coworkers (1, 19). These investigators concluded that the amount of lysozyme present in serum contributes in a striking fashion to spheroplast formation of Gram-negative microorganisms. Metzger et al. have also reported that lysozyme enhances the immobilization of Treponema pallidium by antibody and C’ (28).

It may therefore be pertinent to mention some of the criteria to be fulfilled in the identification of C’ as a participant in these reactions. On the premise that the C’ requirements in these experiments will parallel those for immune hemolysis as discussed by Mayer in this symposium, the cytotoxic assay system will consist of the following reagents, each of which must be carefully standardized for optimal activity. These are the tumor cells, guinea pig serum or other source of C’, antibody, and an adequate level of necessary co-factors, such as divalent cations. To minimize the likelihood that antibodies present in the serum used as a source of C’ might potentiate the cytotoxic activity of the immune serum, the former should be absorbed with the cells under test prior to use. The requirement for divalent cations in the action C’ 1 and C’ 2 has been amply demonstrated (20, 33).

It might, therefore, be anticipated that the enhancement of antibody toxicity for lymphoma cells as reported in (57) could be demonstrated even more effectively had the diluent contained these cations at optimal levels. The presence of citrate or phosphate in the buffer used as diluent in these studies may have led to still further difficulties when fresh guinea pig serum was added in varying amounts as a replacement for the buffer. Under these conditions, the cation levels were not uniform in the different tubes of the titration series, thus possibly obscuring sharp end-point determinations. Verification of this effect can be established by hemolytic assays of the reaction mixtures, which should comprise an essential control for this type of study. The activity of the antibody-C’ system in destroying tumor cells should be checked for residual C’ by means of hemolytic titrations. Assurance would thus be provided that the neutralization end point was not curtailed because of a C’ deficit in the assay reaction mixtures.

Many investigators have relied solely on the differential action of fresh and heated sera for C’ identification. The inadequacy of this criterion may be emphasized in the statement that some antibodies to tissue and other antigens, especially those present in so-called normal sera, are also destroyed in large measure by the thermal inactivation conditions (56°C, 30-min.) used to destroy the heat-labile C’ components, C’ 1 and C’ 2 (27, 36, 37). Specific decomplementation—i.e., removal of C’ components by absorption with a specific precipitate formed by an unrelated immune system—constitutes an additional experimental maneuver of value to this type of study provided titrations establish the simultaneous loss of hemolytic and neutralizing activity.

The recent demonstrations that salicylaldoxime and phlorizin act as specific inhibitors of one of the third C’ components provide still another means for verifying the assumption of C’ participation (30, 49). Finally, in systems which utilize the mouse as the test animal for cell viability, correlation of in vitro with in vivo effects of C’ should not
prove a serious deterrent, since the total lytic activity for sensitized erythrocytes in the mouse is probably equivalent to 0.1 ml. of undiluted guinea pig serum. In consequence, the addition of guinea pig serum may be expected to lead to marked increases in neutralizing potency as demonstrated in (57) provided anticomplementary effects do not intervene and the evidence for a C' requirement is otherwise valid. The current availability of improved methods for preparation and assay of individual C' components will greatly facilitate studies along these lines in providing additional criteria for unequivocal identification of C' in terms of well characterized intermediates of immune hemolysis (see article by Mayer, this volume and chapter on C' in [20]).

D. OTHER STUDIES

Finney and co-workers have recently renewed the claim that the serum of patients with terminal malignancy may contain cytotoxic antibodies to their own tumors (8, 9). The antibody response was estimated by hemagglutinin titrations with tanned sheep erythrocytes. Although the technical details of antigen preparation for coating the red cells are not given, it may be assumed that these were homogenized extracts of autologous tumor tissue. Similar preparations, derived from the patient's own tumor tissue and incorporated in complete Freund's adjuvants, were used for active immunization. Many of the patients also received hyaluronidase in the injection material in order to minimize the formation of debilitating abscesses. There are several aspects of these studies which require further consideration before these experiments can provide definitive evidence for auto-antibody production in human cancer.

The data of the present experiments indicate enhanced hemagglutination activity following injection of the patient with extracts of his own tumor tissue. The tumor tissue used for inoculation undoubtedly contained an undefined mixture of antigenic substances from normal and neoplastic tissues. The assumption that the serologic reaction was mediated only by the latter cannot be accepted without further evidence. Controlled studies with various normal tissues derived from the same or other patients are indicated to provide a basis for estimating organ and individual specificity and to preclude the interplay of viral or bacterial antigens in these experiments. We have observed that rabbits given injections of complete Freund's adjuvant may respond with the production of Wassermann antibody. Further, sera of patients with a variety of nonmalignant diseases can react with alcoholic (34) and aqueous extracts (10) of normal tissues. The studies of Waksman et al. (55) and of Pearson (41) clearly demonstrate that Freund's adjuvant is not an inert mixture. In rats, a disseminated inflammatory reaction affecting many tissues may be produced following injection of killed tubercle bacilli emulsified in oil. These animals also showed marked skin reactions to tuberculin which were attributed to dissemination of mycobacterial antigens. In view of these findings, future studies of auto-antibodies in cancer might include immunization with incomplete adjuvant and hemagglutination studies with the patients' sera after absorption with normal and neoplastic tissues, as well as with heat-killed tubercle bacilli, if these were present in the inoculation material. The incorporation of hyaluronidase, penicillin, and streptomycin introduces further interpretational difficulties in that at least two of these preparations contain several antigenic components of potential significance to the hemagglutination reaction.

The observations drawn in these studies (8, 9) that an increase of hemagglutinating antibody may also follow deep x-ray therapy would seem to vitiate the objections noted in the active immunization experiments. A detailed confirmatory report would provide a firmer basis for judgment. However, the possibility must still be entertained that extensive irradiation and accompanying tissue damage may effectively release an intracellular component which normally is but feebly antigenic, if at all. Reference is made to cardiolipin which has been demonstrated in human tissues by means of fluorescent antibody studies (21) and which is markedly antigenic only in syphilis. The cellular constituents reactive with sera in lupus provide another such illustration. Finally, the possibility must be eliminated that the hemagglutination reaction involved a nontumor cell antigen that may have been adsorbed to the sheep erythrocytes from the homogenized tissue extracts. This type of contamination has been noted in the case of bacterial agglutination due to the adsorption of lysozyme onto the surface of bacteria grown in broth enriched with serum. Antibody to lysozyme agglutinated the microorganisms (3).

Among other reports ascribing antigenic specificity to tumors on the basis of anaphylactic experiments are the studies of Makari (25) and of Zilber and his colleagues (53, 60). In the procedure followed by Makari, female guinea pigs are given injections of an extract of tumor tissue. Several weeks later, the uterine horns of this and of a non-immunized animal are placed in the same Schultz-Dale bath and tested for reactivity with normal serum. Following this treatment, the serum of the
patient from whom the tumor was derived or an extract of the tumor is added to the bath fluid. In the author's hands a greater contraction of the uterine muscle from the immunized pig was observed in more than 95 per cent of cancer patients. The potential utility of this approach, confirmed by Burrows (6) and denied by Hackett and Gardonyi (17), invites critical appraisal. Many questions of a theoretical nature can be raised concerning the validity of these findings with respect to the untold numbers of antigens present in the crude tissue extracts, the requirement for the presence of the tumor-specific antigen in the patient's serum, the failure to control many aspects of the procedure, the possible involvement of blood group and plasma protein antigens, and the difficulties encountered by the author in dealing with autologous material.

In an effort to surmount some of the technical barriers associated with this procedure, the writer and his colleagues have developed an isometric apparatus for the study of antigen-antibody interaction in guinea pig smooth muscle. Technical details of the procedure are given in (32, 43). The method utilizes 30–40 segments of guinea pig ileum, thus providing ample opportunity for replication of tests and for evading the problem of individual variability. Measurements of reactivity to histamine, serotonin, and to immune reagents with sensitized tissues yield a standard error of the mean approximating 10 per cent. Data typifying the response of the passively sensitized guinea pig ileum to increasing levels of homologous antigen (egg albumin) are given in Chart 6, in which the results of four experiments are summarized. The tracings which provided these data were also analyzed for the duration of the latent period which begins with the addition of antigen to the bath and which terminates with contraction of the tissue. These data, given in Chart 7, show the inverse linear relationship which characterizes the reaction in the region of partial response. With further increments of antigen, the latent period approaches a minimum of about 10–15 seconds for this immune system. It was of interest to note that the minimal latent period for histamine is about 1 second, whereas that for anaphylatoxin, a physiologi-
cal mediator of histamine release, is about 1 second. Further exploration of the factors which influence the duration of this initial phase of smooth muscle contraction, and of the events at the cellular level, are required. The data show that careful measurements of this interlude provide an additional and sensitive estimate of the response of sensitized tissue to specific antigen.

It may be anticipated that the degree of reproducibility and replication attainable with this assay system can be helpful in the identification of tumor-specific antigens. Since the method permits the use of 30 or more intestinal segments from a single guinea pig and requires minute quantities of reagents, it becomes possible to carry out all the necessary control experiments and to replicate the observations with the tissues of a single animal. This degree of flexibility in the experimental design should prove valuable in attempts to confirm the studies reported by Zil'ber on systemic anaphylaxis in guinea pigs (53, 60). In these experiments, it would be of interest to compare the results obtained with tumor-specific antigens. Since the method permits the use of 30 or more intestinal segments from a single guinea pig and requires minute quantities of reagents, it becomes possible to carry out all the necessary control experiments and to replicate the observations with the tissues of a single animal. This degree of flexibility in the experimental design should prove valuable in attempts to confirm the studies reported by Zil'ber on systemic anaphylaxis in guinea pigs (53, 60). In these experiments, it would be of interest to compare the results obtained with tumor-specific antigens. Since the method permits the use of 30 or more intestinal segments from a single guinea pig and requires minute quantities of reagents, it becomes possible to carry out all the necessary control experiments and to replicate the observations with the tissues of a single animal. This degree of flexibility in the experimental design should prove valuable in attempts to confirm the studies reported by Zil'ber on systemic anaphylaxis in guinea pigs (53, 60). In these experiments, it would be of interest to compare the results obtained with tumor-specific antigens.

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Immunologic Studies of Autochthonous Cancer An Evaluation of Several Procedures

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