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DISSERTATION

Presented in Partial Fulfillment of the Requirements for Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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The Ohio State University 1967

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INTRODUCTION

Ever since the time of Paul Ehrlich, many attempts have been made to apply immunologic theories and methods to produce a better understanding of cancer. The voluminous literature in this area (See reviews by Old and Boyse, 1964; Hattler et al., 1966) has provided substantial experimental evidence for the fact that tumors contain antigens not found in the other cells of the body. Much of the earlier characterizations of tumor antigens were done with extracts of malignant tissues. Antisera prepared to these extracts were absorbed with normal tissue in order to remove anti-normal tissue reactivities and subsequently were considered to be tumor specific. However, in the interpretations of these experiments, little attention was paid to the fact that some antigens may have been masked, altered, destroyed or lost in the initial extracts. By far, the most serious flaw in this approach was the use of malignant and normal tissue from different individuals. Hence, some of the antibodies labeled "tumor specific" in these studies were, in reality, the result of individual antigenic differences between donors.

The present information concerning the cellular control mechanisms has made it abundantly clear that neoplasia is an escape from normal growth control mechanisms. Evidence for specific interaction of carcinogenic agents and cellular nucleic acids has given added impetus to the above concept. Such interaction of antigenic agents, either viral nucleic acid, chemical carcinogens or radiation, may
possibly lead to an increase in the range of de-repression in the cell which may either be expressed in an overall regulation of cell metabolism, in the synthesis of new protein or both. Certain immunological evidence indicates that malignant transformation is accompanied by the loss of substances capable of acting as antigens. This evidence provides powerful support for the enzyme deletion theory of carcinogenesis. Equally convincing data have been reported demonstrating the appearance of new antigens in cancer cells.

The present work was undertaken to demonstrate the presence of antigenic differences between normal and malignant human tissues obtained from the same human donors. Various cellular organelles isolated by refined biochemical techniques were used to examine the antigenic loss or gain in the cancerous tissues. Particular consideration was given to the detection of antigenic differences at the nucleic acid level.
CHAPTER I
REVIEW OF THE LITERATURE

For some seventy years, immunologists have been trying to answer a key question: whether cancer cells possess or do not possess characteristic antigens? There is no doubt that cancer cells, like any normal cells, contain various kinds of antigens such as species and organ specific antigens, blood group substances, histocompatibility factors, and heterophile antigens of the Forssman type. However, by combined use of immuno-chemical and biochemical methods numerous investigators have recently provided evidence to show antigenic differences between tumor and normal cells. The precise nature and significance of these antigenic differences are still disputed, because in a number of cases these differences are quantitative rather than qualitative. True cancer specific antigens are rare, but do exist, and two classes, those of virus induced or chemically and physically induced tumors, have been carefully documented and studied in some detail (Hellstrom, K. E. et al., 1965; Old, L. J. et al., 1964; Sjorgen, H. O., 1965).

Tumors are caused by a variety of chemical, physical, and biological agents. Each kind of tumor has a peculiar antigenic structure depending upon its causative agents. In the case of virus induced tumors, they might contain complete or incomplete virion. In chemically or physically induced tumor, there may be interactions between carcinogenic substances and metabolites of the cell. Finally in transplanted tumors, there may occur isoantigens due to genetic differences between host and grafts.
Because of the unique antigenic structure of tumors of different origins, this review has been divided into three principal parts: (1) antigens of virus induced tumors; (2) antigens of non-filterable tumors; (3) antigens of human tumors. Another special section is devoted to the role of nucleoproteins and nucleic acids as tumor antigens.

Antigens of Virus Induced Tumors

Two main theories of carcinogenesis have been proposed, the mutation theory and virus theory. The former theory, championed by the Madison school, through Heidelberger and Potter, supposes that cancers result from imperfect duplication of one or more genes in the nucleus of a body cell. A mutation somehow alters the growth characteristics, presumably changes in one or more enzyme system (Heidelberger, 1959; Potter, 1956). The studies of the former on carcinogen-protein interactions, supported by much indirect evidence, suggested a biochemical means whereby specific antigens, proteins or enzyme protein, could be gradually or permanently eliminated or suppressed in successive generations. These studies have shown not only protein-carcinogen interaction, but also nucleic acid-carcinogen interactions.

Current progress in virus research has provided a large amount of experimental evidence concerning malignant growth induced by viruses in experimental animals. As early as 1908, Ellerman and Bang described avian leukosis as being of viral etiology and the years from 1951 to 1959 saw the characterization of several new viral leukemia agents. Among these have been the viruses described by Gross (1951) in A K strain
mice, and later as "passage A" strain in Bittner C3H mice (Gross, 1957). Schoolman et al. (1957) found that lymphocytic leukemia could be produced in Swiss mice which had been inoculated with cell free brain filtrate from Swiss leukemic mice, other studies by Friend, (1957), Metcalf (1958), and Moloney (1960) have shown the association of viruses with different kinds of malignant transformation.

Besides causing different kinds of leukemia, viruses also have been associated with solid tumors, the best known virus being polyoma. Stewart and associates (1957) have shown that polyoma virus produces a variety of solid tumors when inoculated into mice, while Monroe and Vindle (1963) have reported growth of subcutaneous tumors in newborn monkeys inoculated with Rous sarcoma virus. However, adult monkeys did not develop tumors.

It seems logical to assume that the antigenic differences between cells of filterable tumors and normal cells would be due to antigens of the virus contained in the tumor cells. Attempts to isolate these viruses from antigenic components of normal tissues is by no means an easy task. Thus, Rous sarcoma virus obtained by different methods in a highly purified state reacts in precipitation and complement-fixation tests with sera of animals immunized with normal chick tissues (Furth and Kabat, 1941), which makes it difficult by means of these reactions to distinguish it from the antigens of normal tissues. With the advances in newer techniques of isolation and purification, this difficulty has been largely overcome and there are numerous reports indicating that virus induced tumors do possess antigens distinct from those of normal cells. Thus, Habel (1961) and Sjorgen, Hellstrom and Klein (1961)
reported that tumors induced by polyoma virus, despite their varied origin contain distinct tumor specific antigens. Huebner et al. (1964) reported specific complement-fixing viral antigens in hamster and guinea pigs tumors induced by the Schmidt-Ruppin strain of avian sarcoma. Huebner (1963) also described specific complement-fixing antigens in SV40 tumors and transformed cells, distinct from SV40 viral antigens, Old, Boyse and Lilly (1963) described the formation of cytotoxic antibodies against leukemias induced by Frient virus, while Slettenmark (1963) discussed the possible tumor specific antigenicity in leukemia induced by the Gross agents. Klein and Klein (1964) have described the antigenic properties of lymphomata induced by Moloney agents. The tumors proved to be highly antigenic in syngeneic hosts. Extensive cross-resistance was demonstrated between different Moloney lymphomata, but there was no certain cross reaction between them and lymphomata induced by Gross virus or with frequently transplanted lymphomata. It appears, therefore, that lymphomata induced by the Moloney agent differed antigenically from those of other types.

The studies on polyoma virus induced tumors have provided very convincing evidence for the presence of tumor specific antigens. In contrast, tumors produced by the Gross agent or the mammary tumor agents which seem to be associated with spontaneous disease produced only after injection of virus itself in newborn animals, (Sjorgen, 1962). Mature animals (mice) do not develop tumors after infection with virus but demonstrate instead antiviral antibodies, and following infection with virus, both newborn and adult mice can be
shown to be resistant to tumor isograft of polyoma origin (Sjorgen, 1961). It appears according to Sjorgen (1964) that resistance is immunological in that it may be diminished by irradiation before virus inoculation. However, the presence of antiviral antibodies in animals resistant to tumor transplants might indicate that the tumor specific antigens in polyoma tumors are viral in origin.

The review by Old and Boyse (1964) has provided a long list of specific antigens recorded in experimental tumors. They make a sweeping generalization that chemically induced tumors differ widely in their immunizing capacity, ranging from those of high antigenicity to those of such low antigenicity that they are demonstrable only by challenge with tumor cells. In addition, two tumors produced in a single animal by the same component fail to cross react. While chemical-carcinogen-induced tumors possess individually distinct antigens, tumors induced by a given virus contain the same cross reacting cellular antigens.

From the foregoing account, it seems that in most virus induced tumors, the virus represents an additional antigenic factor. Whether or not it retains its antigenicity distinct from host antigen or combines with and in some way changes the host antigen profile (such as DNA or RNA) has not been shown conclusively.

Antigens of Non-Filterable Tumors

Non-filterable tumors can be divided into two major categories: (i) primary tumors induced by various carcinogenic substances and treatments; (ii) transplantable tumors. Besides these, there is another
special group of tumors which are of spontaneous origin in animals and humans, the etiology of which is not known.

Antigenic analysis of transplantable tumors is usually carried out in highly inbred strains of animals in order to avoid, as far as possible, the isoantigenic differences between donor and recipient which may be falsely interpreted as tumor antigens. The most widely studied antigens in transplantable tumors are those of the histocompatibility antigens. Organ specific antigens are often located inside the cells and only in certain situations do they function as histocompatibility antigens.

Studies by Amos (1963) and his co-workers have shown that histocompatibility antigens are present in varying amounts in normal tissues and in neoplasms arising from these tissues. They are generally responsible for the failure of a transplanted tumor to take in an antigenically dissimilar (allogeneic) recipient. Snell and co-workers, in a series of publications, have reported on mouse histocompatibility antigens. They have shown that in mice these antigens are controlled by genes at a number of loci, but chiefly by genes at a locus called H-2 (Snell, 1953). Gorer (1956) has shown that antigens of the H-2 locus are responsible for the prompt rejection of grafts carried across H-2 barriers. Histocompatibility antigens are not confined to mice but have been shown to exist in other species, and recently in man (Batchlor, 1965). Because of the histocompatibility barrier, demonstration of antigenicity of transplanted tumors has to be carried out in isologous hosts.

Gross (1943) was the first to use inbred animals to study the antigenicity of methylcholanthrene (MC) induced tumors. Mice of C3H inbred
line were first inoculated with 0.01 to 0.03 ml of a 20 per cent tumor suspension from a C3H induced tumor. One hundred fifteen mice received tumor cell suspensions. In 91 animals the intradermal tumors grew progressively and eventually killed their hosts in 41 to 85 days after inoculation. In 21 mice the intradermal tumors persisted only temporarily, then gradually regressed and disappeared. When rechallenged with the same tumor, these animals showed a high degree of resistance as compared to controls. These studies showed that an immunity was specifically directed against the tumor.

Lewis in 1951 and Foley (1953) demonstrated the existence of tumor specific antigens in methylcholanthrene induced mammary carcinomas in C3H/He mice. Subsequent work of Prehn and his associates (1960) demonstrated isologous immunity in dibenzanthracene induced carcinomas in C3H/He and BALB/c mice. In a later study, Prehn (1962) showed that methylcholanthrene induced adenocarcinomas elicited tumor specific immunity when transplanted to isologous mice. Subsequent studies by Old and his associates (1962) demonstrated that tumors which appeared first after induction were highly antigenic, whereas those which appeared later were weakly antigenic or non-antigenic with respect to tumor specific antigens.

Most of the work on demonstration of tumor specific antigens in transplanted tumors has been summarized in a number of recent reviews (Prehn, 1962; Old, 1964; Prehn, 1963). From the extensive work done in this area, one important point emerged, that in many cases differences are quantitative rather than qualitative, i.e. that the apparent specific
tumor antigenicity represents a tremendous increase in normal, but otherwise undetectable, antigens. This possibility also influences thinking on tolerance and the failure to show immunity in autochthonous hosts in some circumstances.

Much of the work on animal tumors has been carried out by comparing antibodies of animals immunized with tumor tissue, or with its various components, with those of animals immunized with normal tissues. These studies have indicated significant differences between normal and malignant tissues.

Kidd (1938, 1940, 1946) studied in detail the antigenic properties of Brown-Pearce rabbit carcinoma. Saline extracts prepared from tumor tissues of rabbits fixed complement with sera of rabbits of blue cross strain implanted with this tumor. However, no reaction was obtained with extracts from tissues of normal kidney, liver, spleen, and red bone marrow, or pus of various origins as well as from tissues of other rabbit tumors. It was concluded from the extension of these studies that Brown-Pearce tumors contain an antigen which differed from those of normal tissues which was probably ribonucleoprotein (HacKenzie and Kidd, 1945) combined with microsomes of tumor cells.

Hoyle (1940) showed that alcoholic extracts of mouse tumors, but not of normal tissues, exhibited a complement-fixation reaction with sera of mice in which tumors had been grown. The reaction was negative with control sera. An ether and alcohol soluble lipid antigen has also been revealed in other transplantable and some spontaneous tumors as well as in mitochondria of rat lymphosarcoma (Rapport and Graf, 1954, 1955). Miller (1955) examined the alcohol extracts of rat sarcoma and
lymphoma and showed that the extract contained protein immunologically different from the protein of alcohol extracts of normal muscles of the same strain of rats.

Dulaney (1949) used the complement-fixation test for studying the antigenic composition of cytoplasmic fractions (mitochondria and submicroscopic particles) of leukemic and normal tissues of inbred strains of mice with rabbit antisera to these fractions. There were only quantitative differences in reactivity between leukemic and normal tissues. Similar results were reported by Nungester and Fisher in 1954 for mouse lymphosarcoma.

The work of Angelletti and co-workers (1960) on seven tumors in various strains of mice indicated that the proteins of these tumors closely resembled one another. They used DEAE-cellulose chromatography to fractionate soluble protein from rhabdomyosarcoma, mammary gland carcinoma, and lymphosarcoma in Swiss mice. They found that a large portion of muscle protein from normal mice did not bind to the ion exchange column representing protein of zero or positive charge. The rhabdomyosarcoma protein differed from the muscle protein from which it was originally derived in that 15 to 20 per cent of the total recovered rhabdomyosarcoma protein was not bound to the column. This difference may be due to the fact that some highly specialized proteins of normal muscle were missing in the tumorous tissues.

Witebsky and co-workers (1956) were able to show biochemical differences between normal and malignant thyroid tissues. Ultracentrifugal analysis showed that the peak of thyroglobulin in normal gland contained 58.3% of the component, while the primary and metastatic tumors
consisted of only 4.2% of thyroglobulin. Electrophoresis showed qualitative differences between the normal gland and the metastatic tumor. Witebsky (1956) et al. also carried out immunological studies on these proteins by using the complement-fixation test. They showed that with antisera to the antigens of normal and malignant thyroid tissue that titers for the homologous (tumor) antigen were 10 times those to the normal thyroid antigen. However, the two antigens cross reacted, and the common antibody could not be removed by absorption without greatly reducing the titer for the thyroid tumor antigen.

Weiler (1954, 1955, 1956) showed that microsomes and mitochondria of rat liver cancer induced by Buttergel lack any capacity to react with organ-specific antihepatic antibodies. Similarly antigens have been detected in hepatoma tissues which were absent in healthy liver. However, they are not considered as specific tumor antigens since they have been found in healthy organs as well. Weiler (1956) had, however, shown that organ specific antigens disappeared from cells undergoing carcinogenesis prior to their transformation into cancerous cells. Similar data have been obtained in cancer of kidneys induced by Stilbelstrol (Weiler, 1956).

Korngold (1957) reported extensive studies on tumor antigens by Ouchterlony technique of gel diffusion. He, however, emphasized that it is difficult to determine the lack or gain of antigen in certain tissues by this technique, since the concentrations of antigens vary from tissue to tissue. Moreover, normal and tumor tissues from different individuals do not contain antigens in the same concentrations. Consequently, in comparing the antigen content of a tumor with that of normal tissue, the same individual should be used. In general, it is
easier to show altered protein structure or abnormal protein synthesis than complete lack of an antigen by double diffusion gel technique. Korngold and Pressman (1954) could not show any antigenic differences between lympho-sarcoma tissue and normal lymphoid tissue by gel diffusion technique.

Narcisssov and Abelev (1956) reported antigenic differences between subfractions of normal and tumor tissue in 9, 10-dimethyl-1,2-benzanthracene induced tumors in rats. Mitochondria and microsomal fractions were isolated from tissues and tested against sera of animals having tumors by complement-fixation tests. Positive reactions were obtained with mitochondria and microsomal fraction from tumors of the same animals. Zilber (1955) separated nuclei, mitochondria and microsomes from the homogenate of rat M-1 sarcoma and dissolved each subfraction in weak alkali and then neutralized with acetic acid. All preparations thus obtained were tested by complement-fixation with sera from rats having M-1 sarcoma. The serologic reactivity was greatest in the mitochondrial and microsomal proteins.

In a series of excellent papers, Day, Hiramoto, Yagi and Pressman (Day et al., 1959; Yagi and Pressman, 1961; Hiramoto et al., 1959) reported an investigation on antigens responsible for the combination of antibodies in antisera to Murphy-Sturm rat lympho-sarcoma. The localization of the antibody depended on the distribution of fibrinogen in vivo. The specificity of this tumor antibody was shown by the fact that I^131-labeled antibody against lympho-sarcoma of rats localized mainly in tumors. Sera of rabbits immunized with normal organs contained no such antibodies. Then, Day, Planinsek, and Pressman (1959) demonstrated
that cancers in general differ from normal areas in their fibrin content, and thus, in this sense, fibrin may be considered as a cancer distinctive substance. They also showed that radiiodinated antibodies against, the transplanted Murphy-Sturm rat lympho sarcoma localized in tumors by fibrinogen and localized, at the same time, fibrinogen was deposited as fibrin. The demonstration of antifibrin antibodies in heteroimmune serum was not brought about solely by the fibrin that accumulated in immunizing tumors by deposition from the circulation. Even heparinized tumor cells from the ascites form of the Murphy-Sturm lympho sarcoma which had been washed a dozen times in heparinized Ringers solution gave rise to antifibrin antibodies when injected into rabbits.

Using complement-fixation, Rapport and Graf (1957) characterized tumor distinctive lipids in lipid extracts and washed mitochondrial fractions of tumors, techniques which completely removed anticomplementary reactions. Another precaution taken by these workers was to use three-dimensional titrations, in which both the antisera and the antigen were serially diluted to determine the optimal concentration of the reaction, since the use of a single concentration of either antigen or antiserum may introduce serious errors in complement-fixation titrations. Antisera produced in rabbits by injecting the mitochondrial fraction of rat lympho-sarcoma (Rapport and Graf, 1955), and the extracted lipid from such tumors and from tissues with chloroform-methanol, were non-reactive with tissue extracts from man and several other species of experimental animals. Whole tissue extracts of rat adrenals, brain, kidney, and other tissues, were also
not reactive, although mitochondrial fractions of rat kidney, liver and testes were positive in the tests. Rapport et al. (1958) studied the chromatographic fractions of a rat lympho-sarcoma hapten obtained by chloroform-methanol extraction and determined that the active fraction was found to require the addition of lecithin or cholestrol for specific complement-fixation. Antigenic activity roughly paralleled the content of the ninhydrin positive, phospholipid fraction.

Antigens of Various Normal and Malignant Human Tissues

Numerous studies have been carried out to demonstrate the occurrence of specific tumor antigens in human tissues. These studies have been carried out to a large extent by comparing the capacity of sera of animals immunized with tumor or tumor extracts or various preparations from tumors and normal tissues to demonstrate antigenic differences.

Witebsky (1929) and Hirschfeld (1929) prepared alcohol extracts of various tumors and normal organs and prepared heteroimmune sera in rabbits. Using complement-fixation, they showed that sera would react only with alcohol extracts of tumor but not of normal tissues. However, those sera reacted with red cells and when absorbed by red cells ceased to react with alcohol extracts of cancerous tissues as well. Thus, they concluded that antibodies of the sera belonged to group specific, but not cancer specific antibodies (Witebsky, et al., 1956). An earlier report by Witebsky, however, indicated the occurrence of a specific antigen in human cancerous tissues. He used boiled extracts as antigen because he noted that an antiserum against raw tumors cross reacted heavily with normal tissues. To control blood group differences he used
the tissues from individuals of the same blood groups. From these earlier studies a cancer distinctive lipid was demonstrated in most human cancerous tissues. Studies by Rapport et al. (1959) further characterized immunologically and chemically these cancer distinctive lipids. They fractionated alcoholic extracts of human lung carcinoma chromatographically and obtained an active hapten which was different from the one isolated from lymphosarcoma. The fraction plus lecithin and cholesterol was assayed by complement-fixation using antihuman reticulum cell carcinoma antiserum. The authors named this human lipid hapten cytolipin H and they further observed the cytolipin H strongly reacted with antisera to human carcinoma, including those of lung, breast, kidney, myelogenous leukemia and malignant melanoma, while most normal tissues had little capacity for such reactions. However, for the production of antibodies auxiliary lipids or serum had to be added, since pure cytolipin H did not stimulate the formation of antibodies, and, as usual, that protein moiety was necessary for the production of antibodies. Later studies by Graf and Rapport (1960) showed that cytolipin H was not related to cardiolipin (Nassermann Hapten) and Shapiro et al. (1964) accomplished the total synthesis of cytolipin H.

Numerous techniques have been tried to separate tumor specific substances and study them immunochemically. Genetron (a fluorocarbon, trifluorotrichloroethane), which was originally used to separate non-viral protein from virus-tissue extracts, has also been used to attempt to separate tumor specific antigens from other tissue antigens. Taylor et al. (1959) examined such extracts from human metastatic
ovarian sarcoma, human carcinoma HEP2, Rous sarcoma of fowls and transplantable chicken fibrosarcoma. Crude extracts of human tumors cross-reacted with antisera to all human tumors, as did the three chicken tumor extracts. However, tumor tissue of the two different species did not cross react with heterologous antisera. But, Genetron extraction and ultracentrifugation resulted in virtual elimination of cross reactions between these antisera.

Such extracts of tumor tissue have also been used by other workers. Suspensions of HeLa and Jlll cells grown in tissue culture, and normal tissue, such as uterus removed by surgery, have been extracted with Genetron by McKenna et al. (1962). The numerous crossreactions with antisera obtained with crude antigens were compared with Genetron purified antigen. A marked increase in the specificity of complement-fixation reactions was observed by means of absorption with crude and Genetron treated extracts of uterus and HeLa cells and a specific antigen for HeLa cells was demonstrated as well as common antigens showed by HeLa cells with normal uterus.

Genetron treatment was also found to separate two main fractions of human leukemia and tumor cells, when the protein fractions thus obtained were tested by double gel diffusion technique (DeCarvalho, 1960). While several antigens were shown to be associated with normal components, only one antigen could be found in highly purified extracts of leukemia and tumor cells.

Komgold (1956) initiated the systematic study of human cancer tissue antigens by the gel diffusion technique of Ouchterlony. Screening 46 surgical specimens of normal and malignant human tissues,
Korngold found that one antigen (which he called Antigen 1) was present in all carcinomas of the cervix and uterus, but absent from most normal uterine tissues. Thus, it was considered group specific antigen for cancers of these areas.

Antigenic differences in normal and tumor tissues of the same patient by precipitation in gel of water soluble antigens have been shown by Itakura (1963). After absorption with normal tissue antigens, antisera against tumor tissues showed significant precipitation bands and reaction with the same tumor antigen. Antisera against normal tissues precipitated normal tissue antigens after absorption with tumor antigens. Seligman et al. (1955) compared the antigenic composition of leucoblasts and of normal human leucocytes by gel diffusion technique. They demonstrated that leucoblasts lack one of the antigenic components invariably present in normal leucocytes but failed to reveal in the leucoblasts any antigens which were absent in normal leucocytes.

Bjorklund and Bjorklund (1957) initiated a systematic study of cancer antigens by using lipoprotein extracts of pooled, human malignant, epithelial tumors obtained at autopsy. The extract supposedly contained concentrated cancer antigen and was used to immunize horses. The resultant antisera reacted with HeLa cells in stationary tube cultures. The 1:20 dilution of antiserum caused retraction of cell ectoplasm and cessation of surface activity. Fresh culture medium failed to induce growth of cells treated with a 1:20 dilution of antiserum for 24 hours while some growth was observed with cells treated with the 1:40 dilution. It was also found that HeLa cells had absorbed
the cytotoxic antibody from the antiserum, since absorbed antiserum was nontoxic to HeLa cells. Moreover, the pooled tumor antigen could absorb cytotoxicity from antiserum, but a pool of 1/4 normal human tissues failed to absorb cytotoxicity. The antibody absorbing capacity of the tumor antigen was found to be inactivated completely at 70°C after 60 minutes.

Partial characterization of this antigen was attained by the fact that antigenic activity was not affected by digestion with nucleases, but digestion with proteolytic enzymes resulted in complete loss of activity, indicating that protein was involved. The antigen was also heat labile. Partial loss of activity occurred in one hour at 40°C, and complete loss at 70°C (Bjorklund et al., 1957).

Bonatti et al. (1965) analyzed in detail the antigenic composition of hundreds of gastric tumors of epithelial origin. They separated the carcinomas in various fractions, viz. nuclei, mitochondria, mixed microsomal fraction and ribosomes. These fractions were further analyzed for enzymatic composition and used for immunization in rabbits. The resultant antisera were absorbed with normal organs, plasma (human), blood group substances, and then examined by precipitation and hemagglutination techniques. Different antigenic components were demonstrable in each fraction. However, some of the specific antisera reacted with embryonic tissue.

Makari and Huck (1955) used the Schultz-Dale test to examine 707 serum samples from cancerous patients and sera from 111 healthy individuals. The guinea pigs uteri were sensitized actively of passively with antigens from HeLa cells, from tumor suspensions, and
from supernatant from tumor suspension obtained by centrifugation between 3200 and 10,000 rpm. The healthy controls showed only 6 positive reactions to sensitized guinea pigs uterus. Test sera revealed that there was a common antigen for carcinoma, since it was present in carcinomas of tissues and blood from untreated carcinomas patients, regardless of site of origin or type of carcinomas.

Burrows (1962) examined the sera of 500 individuals with the Schultz-Dale technique of Makari, and obtained essentially the same results. The sera of 96.7% of the patients without carcinomas were negative. There were 10 false negatives in the first group and 7 false positives in the second group. However, accuracy was reduced to only 77% positive in cases showing small early lesions.

There are indications that there is a common antigenic component between tumor and erythrocytes of the host. Bogden and Aptekman (1953) found that the isologous tumors, spontaneous or carcinogen induced, when implanted in Lewis inbred strains of mice, caused a decrease in hemagglutinin titers of host serum for human erythrocytes. There was an initial drop in the titer for AB and O erythrocytes for 48 hours after tumor implantation, followed by a rise in titers for 6 to 9 days, after which titers again declined and completely disappeared in 21 days. Aptekman and Bodgen (1956) later reported that all the reactions of Lewis serum with human AB and O red cells, were dependent on antigens common to the human erythrocytes and not related to A, A and O group specific substances. Adelsberger (1951) demonstrated that heat-inactivated rabbit antisera to mouse mammary tumors hemolyzed erythrocytes from C3H tumor susceptible mice in dilutions of 1:16 to 1:256.
Erythrocytes from C57 black mice were not hemolyzed by the antiserum. Moreover, erythrocytes from normal C3H mice were more sensitive to hemolysins in mammary tumor suspensions than erythrocytes from C57 mice, while erythrocytes from tumor bearing C3H mice were much less susceptible to lysis by tumor extracts. It appeared that an antibody present on the surface of the latter erythrocytes was responsible for inhibiting the action of tumor hemolysins.

Mention has already been made of the work of Aptekman et al. (1956) showing the effect of tumors on the hemagglutinins to human erythrocytes in the plasma of rats. In these studies it was found that the activity of normal rat serum for Group A erythrocytes was related to specific anti-A, while the effect on erythrocytes of the other blood groups was due to another agglutinin (anti-x), which was unrelated to any of the major blood group antibodies. These authors in their later studies (Bogden and Aptekman, 1957) prepared concentrated ethanol extracts of the following tumors, occurring spontaneously or carcinogen induced, in the P.A. strain of rats: sarcoma 6, carcinoma 5 and sarcoma 231. These extracts all inhibited the hemagglutination of human A and B erythrocytes by normal rat plasma. After dialysis of the tumor extracts, their ability to neutralize anti-A and anti-x activity was still apparent, although neutralization of anti-x activity was found to be reduced. The neutralizer of anti-A, which was not dialyzable seemed peculiar to rat tumor extracts. It was of interest that normal rat tissues lacked antigenicity in precipitin reaction while ethanol extracts of lymphoma and sarcoma were antigenic in rabbits.
It seems significant to interject at this point Green's (1957) observations of the causes of anemia in cancer. He observed that the life span of erythrocytes was diminished often by one-half or more in human cancer, and that the degree of anemia was not correlated with replacement or destruction of hematopoietic tissues. By using Coomb's reagent (in greater concentration than that used for the standard antiglobulin test) he found that 53% of general cancer cases tested gave positive erythrocyte agglutination as compared to 3% for normal individuals. He concluded that the cancer patient's erythrocytes were more reactive with antihuman globulin antibody and that this reactivity increased proportionately with tumor mass. He stated that anemia in cancer is due to an immunological mechanism namely, by hemolysins present in the serum. He concluded that (1) antibody is produced in response to tumor antigens which were very close antigenically to erythrocyte antigens, and the antibody cross reacted with them; or (2) the tumor itself was deficient in an antigen and therefore actively produced antibody directed against the host cells. He demonstrated later (1957) that tumor extracts show hemolytic activity (usually heat stable) and hemagglutinating activity (often increased after heating). He also found that direct injection of erythrocytes into tumor tissue, or even in another area of tumorous animals, resulted in a quantitative increase of both the hemolytic and hemagglutinating properties of tumor extracts. This occurred with injections of homologous and also autologous erythrocytes. Hemolytic and hemagglutinating activities accumulated in tumors more than in the spleen; were found to be species specific; and were associated with tumor lipoproteins and phospholipids.
Both transplanted and spontaneous tumors reacted in the same way to erythrocyte challenge. The lipoprotein fraction, however, stimulated strong hemolytic activity, while phospholipids caused the production of strong hemagglutinating activity. It would be interesting to correlate the antigens of tumor extracts reported by Green, and cytolipin H from human cancer investigated by Rapport by complement-fixation.

Deoxyribonucleoprotein and Deoxyribosenucleicacid
as Tumor Antigens

Since the nucleus of a cell controls the gene regulation and cellular differentiation, it seems reasonable to presume that changes from a normal to a neoplastic cell might be brought about at a molecular level in deoxyribonucleoprotein or DNA of the cell. If the model proposed by Jacob and Monod (1961) for the genetic regulatory phenomena in bacteria is widely operative in the cells of other species, then the fundamental problem of oncogenesis is to understand the deficiency in regulation of cell division in cancer. The model postulates that there are regulator genes, the product of which is a repressor substance, and structural genes, which code for the synthesis of enzymes. The repressor, as the name implies, represses the function of the structural gene or genes, presumably by being able to recognize, and react with, a region known as the operator.

It has indeed been shown that the chemical carcinogens specifically interact with nucleic acid structure (Heidelberger, 1959) and that there are distinct morphological irregularities in the chromatin structure of the nucleus in passing from the benign to the malignant
state, Koss and Durfee (1961) have shown that the series of events leading to a cancerous state include nuclear abnormalities from hyperplasia to metaplasia, to dysplasia and finally to a neoplastic product. The work of Kidson (1964) showed that there is an altered pattern of messenger RNA synthesis in mouse hepatoma compared to normal liver. If oncogenic agents such as viral nucleic acids, chemical carcinogens, or radiation acted to either displace histones, promote DNA strand separation, or mimic the nucleotide base sequences of certain normal species of de-repressor RNA, then there will be an increase in the range of de-repression and consequently karyotypic changes (Frenster, 1966).

Such increase in the range of de-repression would be expressed either in an overall regulation of cell metabolism or the synthesis of new proteins. There are some indications that this is the case. Davis and Busch (1960) studied various tumorous and normal tissues, including isolated, fractionated nuclei from tumors in rats with Jensen sarcoma and Flexner-Jobling carcinoma, and from mice with sarcoma 180 and Ehrlich ascites tumor. After injection with labeled glycine, the fractions showed radioactivity with elutions from a similar chromatographic position. This was also observed with a human malignant melanoma obtained by surgery. The same radioactive peak was not found in normal tissues undergoing rapid growth, i.e., embryonic rat tissue and regenerating rat liver. Hence, these findings suggest that significant differences exist between proteins of tumors and other tissues. The authors mention the possibility that nuclear proteins may undergo genetic changes in tumor cells. This change, due
either to mutation of nucleic acid or admixture with viral nucleic acid, takes a direction common to neoplastic tissues. In later studies Byovet and Busch (1961) isolated tumor specific DNA-bound chromosomal protein from rats that was not present in normal cells.

In the case of Balb/c mouse myeloma tumors which elaborate specific globulin molecules which are 20-30% of the total cellular protein, Greenberg and Uhr (1967) have demonstrated by a specific DNA-RNA hybridization technique that differences in DNA's are responsible for specific tumor proteins.

The above evidence suggest that there are differences in the nucleoprotein and nucleic acids of normal and neoplastic cells. If this is the case, then immunoochemical analysis could be applied to detect such differences. This has been the goal of immunologists for a long period of time. But earlier studies concerning the antigenic analysis of nucleic acids were hampered by the poor antigenicity of the material isolated. It is pertinent to quote Landsteiner (1946):

"The experience of poor and wanting antigenicity of animal nucleoproteins which are composed of nucleic acids and histones or protamines is intelligible, since, as evidence hitherto existing would indicate, the protein components have no appreciable antigenic capacity. Bacterial and viral nucleoproteins, on the other hand, were found to induce antibody production. In view of the positive results with some nucleoproteins of animal origin it would be worth while to resume the subject with the use of well purified and unaltered preparations." Some of this difficulty has been overcome by the use of undegraded material and by protecting the nucleic acids from the
action of nucleases so that they reached immunocompetent cells without being fragmented. Numerous workers have been successful in producing anti-DNA, anti-RNA antibodies whose specificities are directed against their constituent nucleotides and nucleosides. Plescia et al. (1961) produced antibodies against DNA and RNA by coupling them with methylated bovine serum albumin while Beiser et al. (1962) have produced antibodies reactive to DNA by conjugating with various nucleotides, nucleosides, and bases to BSA by various chemical means. Antibodies against RNA, ribosomes, and various polynucleotides have been reported by various workers (Dodd et al., 1963; Sela, 1965; Barbu and Paniñel, 1961).

This rapidly expanding area of immunochemical analysis has been the subject of numerous excellent reviews (Beiser et al., 1966; Sela, 1965; and Plescia et al., 1967).

There have been very few reports on the antigenic analysis of DNA-P or DNA from neoplastic and normal cells. Maculla (1947, 1948) prepared nucleoprotein fractions of transplantable mouse tumors and normal organs and showed these were different immunologically. She used the complement-fixation test with sera obtained through immunization of rabbits with various preparations. Antigens of cancerous and embryonic tissues were found closely related. Antigenic differences in the nucleoproteins from rat sarcoma from those of liver, spleen and other organs have been detected by Manciolov et al. (1953).

Zilber (1957), because of his interest in the viral etiology of cancer, presumed that nonfilterable tumors might contain masked viruses which could be obtained from the nucleoprotein fraction, isolated crude nucleoprotein fraction from various human tumors. The fractions
were injected into guinea pigs. After 25 to 30 days, the animals were desensitized by small doses of corresponding normal tissues two or three successive courses of inoculation, until the desensitization to normal tissue was established. Injection of the sensitizing tumor fraction invariably produced anaphylactic shock in the guinea pigs. The conclusion was that the tumor possessed, in addition to antigens to normal tissues, an antigen specific for the tumor. The reaction was obtained with a wide variety of human tumors, including carcinoma of the liver, stomach, mammary gland, uterus, bladder, several sarcomas and leukemic spleen. Zilber and co-workers confirmed these conclusions by demonstrating the converse reaction. This consisted of immunizing the guinea pigs to normal tissues followed by desensitizing with tumor tissues. On subsequent challenge with the corresponding normal tissue no anaphylactic shock was seen. More recently, Zilber (1962) tested these fractions with antisera prepared in rabbits and showed differences by immunodiffusion.

In a series of recent papers, Perez-Cuadrado and Haberman (1964, 1965) and Race and Haberman (1965) used various methods to characterize antinuclear antigens from human malignant tissues. They separated DNA-bound protein from nuclei by differential centrifugation. These fractions were characterized by the demonstration of specific nucleic acid enzymes and spectrophotometrically, and were used to immunize rabbits. The resultant antisera were extensively absorbed with blood group substances, normal organs and normal nuclear antigens and then tested for reactivity with DNA-bound protein from malignant tissue by agglutination with sensitized polystyrene latex particles. They
detected cancer specific antigens in DNA-bound protein from cancer cell nuclei. These findings were further confirmed by immunofluorescence and gel diffusion.

Messineo (1961) introduced a very mild technique of extracting DNA-P from various tissues with 0.1 M glycine. Physiochemical studies of such isolated DNA-P showed that the material was essentially undegraded (Messineo, 1962). He used this DNA-P isolated from normal and leukemic leukocytes to immunize rabbits and the sera were tested by gel diffusion. Distinct immunological differences were demonstrated between deoxyribonucleoproteins of normal and leukemic white cells. The above mentioned results suggest that significant differences can be detected in DNA-P and DNA of normal and neoplastic cells.
CHAPTER II

MATERIALS AND METHODS

Biochemical

Tissues

The tissues used in this study were obtained from human patients in the Department of Surgery, Ohio State University Hospital, at the time of surgery. Both tumor and normal tissues were obtained from the same patient and the tissues were immediately placed in ice after surgical removal. The malignant nature of the tumors was confirmed histologically in the Department of Surgical Pathology, University Hospital.

Tumors and normal tissues were obtained from the following patients:

It should be emphasized that the same donors have been used as a source of normal and tumor material in order to eliminate antigenic differences between individuals (isoantigens). As can be seen from the above list that the major source of tissues were human colon cancer. The specimens removed surgically for primary adenocarcinoma of the colon were taken from various sites ranging from the cecum to the rectosigmoid junction. Adenocarcinoma of the colon is particularly well suited for this type of study because it usually does not extend submucosally more than 6 to 7 cm on either side of the grossly visible tumor (Akerman, 1962). The portions of the specimen more than 7 cm proximal or distal to the cancerous growth could be considered normal tissue. Random microscopic examination of the areas of tissue considered to be normal, by the surgical pathologists, failed to reveal the presence of cancer cells at any time. The portion of apparently normal tissue 7 cm from the tumor either side of the neoplasm was removed and discarded, only the central, obviously cancerous portion was used as tumor tissue.

Isolation of Subcellular Fractions

Before using the tissues for isolation of subcellular particles, the necrotic tissues as well as blood vessels and connective tissues aseptically dissected as far as possible at 4°C. All suspending media contained 1:10,000 aqueous merthiolate as a preservative. The tissues were placed in 2.5 volumes of L.M. medium, consisting of 0.25 M sucrose, 0.065 M KCl, 0.035 M KHCO₃, 0.01 M MgCl₂ and 0.05 M tri(hydroxy methyl aminomethane-HCl of pH 7.8 and were cut into small
pieces with stainless steel scissors. They were then homogenized in the Tri-R homogenizer (Model S63-C, Tri-R Inc., Jamaica, N.Y.) until individual cells could be seen. This was usually accomplished by homogenizing 2 to 5 minutes at 4,000 to 5,000 rpm. The homogenate was then put into an Artisan pressure homogenizer (Artisan Industries Inc., Waltham, Mass.) and exposed to nitrogen under 900 lb pressure for 30 minutes. When the pressure was suddenly released, most of the cells were ruptured. The homogenate was then strained through two layers of cheese cloth and subjected to differential centrifugation as follows: 2000 x G for 10 minutes at 4°C in a Sorvall refrigerated centrifuge to sediment the nuclei. The supernatant was then centrifuged for 60 minutes at 105,000 x G in a Spinco Model L preparatory ultracentrifuge to sediment the microsomes. The microsomes were suspended in a medium of 0.5% deoxycholate (Mann Laboratories) in L.N. medium. A 5% solution of deoxycholate was prepared by placing deoxycholic acid in 0.05 M Tris buffer at pH 8.6 and homogenizing it in a Dounce homogenizer. The pH was then brought to 8.6 with 3 N KCl, and centrifuged at 105,000 x G for 120 minutes. The residue after centrifugation was considered to be ribosomes.

Further Purification of Nuclei

The 2000 x G sediment obtained above was further purified by the method of Chauveau (1956). It was suspended in 9 volumes of 2.2 M sucrose with mild homogenization, and the suspension was centrifuged at 40,000 x G for 1 hour. The supernatant was decanted and discarded.
TISSUES

Homogenized in 0.25 M sucrose
(L M Medium)

Nitrogen Cavitation at 900 lb for 30 minutes
2000 x g for 10 minutes

Nuclei and Unbroken cells

Supernatant

2.2 M sucrose
40,000 x g
(60 minutes)

NUCLEI

Sediment
MITOCHONDRIA
(washed 2 times with 0.25 M sucrose)
105,000 x g
(60 minutes)

Sediment
in 0.5% deoxycholate
in L M Medium
105,000 x g
(120 minutes)

Sediment
RIBOSOMES

Supernatant
(discard)

Figure 1

CELLULAR FRACTIONATION PROCEDURE
The cytoplasmic fragments adhering to the walls of the centrifuge tubes were removed with the aid of clean cheese cloth. The nuclear fraction obtained was 80-90% pure as seen by phase contrast microscopy and by methyl green GA-pyronin stain (Pearse, 1960). The procedure of isolation of the subcellular particles is schematically shown in Figure 1. All extraction procedures were carried out at 4°C.

Subfraction of Nuclei

The isolated nuclei were subfractionated by the method of Steele and Busch (1963) to separate various acidic nuclear proteins. Figure 2 shows the schematic procedure for the isolation of the nuclear proteins fractions. Soluble nuclear proteins were extracted twice with 5 volumes of 0.14 M NaCl once with 2-3 volumes of 0.1 Tris buffer pH 7.6, in that order, by homogenization and stirring for 20 minutes to yield fractions I and II. A precipitate was obtained in each case by centrifugation at 6,000 x G for 10 minutes at 4°C. After this extraction, the precipitate was extracted with 1-2 volumes of 2.0 M NaCl for each gram of wet tissue to remove the deoxyribonucleoprotein Fraction III. The extraction was accomplished by homogenizing the precipitate in 2 M NaCl for 20 seconds at 4°C with Tri-R. The extract was centrifuged at 25,000 x G for 1 hour at 4°C and the clear supernatant solution was decanted and stored overnight at -20°C. The precipitate was then stirred overnight with one fourth of the initial volume of 2 M NaCl with the aid of magnetic stirrer, and reextracted for 1 hour the next morning. The suspension was combined and centrifuged at 20,000 x G for 20 minutes and the supernatant decanted as DNA-P-II. The residue was saved for absorption.
Isolated nuclei

0.14 M NaCl 2 X Homogenized; Centrifuged 6000 x G for 20 minutes

Precipitate

Nuclear sap
Proteins (I)

0.1 M Tris pH 7.6; Homogenized; Centrifuged 6000 x G for 10 minutes

Precipitate

2 M NaCl; Homogenized; Centrifuged 25,000 x G for 1 hour

Precipitate

Nuclear sap
Proteins (II)

Precipitate

2 M NaCl (as above)

Precipitate

DMP-1 (III)

DMP-2

0.5 N H_2SO_4
or 0.25 N HCl
20,000 x G for 20 minutes

Precipitate

(Saved for absorption)

Histone (IV)

Figure 2

PROCEDURE FOR SUBFRACTION OF NUCLEI
Histone (IV) was isolated from DNA-P as follows: The 2 M NaCl extract was thawed at 4°C and treated with 0.25 N HCl or 0.25 N HCl or 0.5 N H₂SO₄ to extract histones and other proteins and centrifuged at 20,000 x g for 20 minutes in a Sorvall refrigerated centrifuge at 4°C. The residue was extracted twice with 0.25 N HCl or 0.5 N H₂SO₄ and was centrifuged again. The supernatant solution was combined and adjusted to a final concentration of 20% trichloroacetic acid. The precipitated protein was sedimented and washed with the same concentration of trichloroacetic acid and then ethanol. The precipitate was redissolved in distilled water and used as Fraction IV. The residue remaining after extraction of histones was saved for absorption of antisera.

Precipitation of DNA-P from the 2 M NaCl Extract

2 M NaCl extracts containing DNA-P were thawed at 4°C. The nucleoprotein was precipitated from the extract by the addition of 2 volumes of cold 95% ethanol during a period of 3 hours with constant agitation. The precipitated fibers of nucleoprotein were removed with a glass rod, washed 2 times with 75% ethanol and redissolved in 0.1 M NaCl. This was coupled to red cells and used as the antigen indicated as Fraction III in the hemagglutination reactions to be described.

Glycine Extraction of DNA-P

It is probable that DNA-P isolated with 2 M NaCl might be heterogeneous and partly degraded because of the high ionic environment and so attempts were directed to the isolation of DNA-P in a low ionic...
environment under mild conditions of extraction. The method suggested by Messineo (1962) and Atchley et al. (1964) seemed to fulfill these objectives. Isolated nuclei from both normal and malignant tissues were dissolved for 1 hour in 0.05 M sodium citrate. Then they were quickly washed with 0.1 M glycine and centrifuged at 7000 rpm for 20 minutes at 4°C. The supernatant fluid was poured off and the precipitate was suspended in 0.1 M glycine (final concentration) and extracted overnight under sterile conditions at 4°C with constant agitation by a magnetic stirrer. The extract was clarified by centrifugation at 24,000 x G for 30 minutes at 4°C. The supernatant fluid containing the DNA-Pr was decanted and preserved under sterile conditions at 0°C. All the steps were carried out at 4°C and 0.001 M EDTA was added to all solutions to inhibit the action of DNA-ase.

Isolation of DNA from 0.1 M Glycine and 2 M NaCl Extracted DNA-Pr

The samples of DNA-Pr were incubated at 37°C for 1 hour with a 1/20 volume of 0.2% sodium deoxycholate (Anagnostopoulous and Spizizen, 1961) and left overnight at 4°C. The precipitate of the protein which resulted was removed by centrifugation at 4°C for 20 minutes at 2000 x G. The crude DNA was collected again from the supernatant by precipitation with 2 volumes of 95% chilled ethanol, resuspended in 2 M NaCl, and the deoxycholate treatment repeated. The ethanol precipitated DNA was suspended in 0.0005 M citrate - 0.1 M NaCl and brought to a pH of 7.0 with a small amount of NaHCO₃. This solution of DNA was used for the hemagglutination tests.
In another procedure according to Jordan (1961) DNA-P was treated with 2 mg/ml of crystalline trypsin (Sigma) at 37°C and left overnight at 4°C under sterile conditions. The solution was filtered through 3 layers of cheese cloth and DNA from the supernatant was precipitated by 70% chilled ethanol, redissolved in 0.14 M NaCl and precipitated with equal volume of 98% ethanol. The fibrous precipitate was then suspended in 0.0005 M citrate - 0.1 M NaCl at pH 7.0 (adjusted by NaHCO₃), and stored at 0°C.

Enzymatic Treatment

Both DNA-P and DNA were treated with DNA-ase and RNA-ase as follows:

DNA-ase 2X crystallized (Nutritional Biochemical Corp., Cleveland, Ohio) was used in a 0.1 mg/ml concentration to treat the substrate (DNA-P) at 37°C for 1 hour after it was adjusted to pH 5 with 1 M acetate buffer and 0.05 M magnesium sulfate. The solution was brought back to pH 7.0 with NaHCO₃ and centrifuged. The supernatant was used as DNA-ase treated antigen.

DNA and DNA-P were treated with 5X crystallized ribonuclease (NEC) at a concentration of 0.1 mg/ml at a neutral pH at 37°C for 2 hours. DNA-P or DNA was then precipitated with chilled ethanol, redissolved in 0.005 M citrate - 0.1 M NaCl to be used as antigen.

Antigens were treated with trypsin (2X crystallized - Sigma) in a concentration of 0.1 mg/ml in phosphate buffer pH 7.6 at 37°C for 60 minutes. DNA-P and DNA was precipitated with two volumes of 70%
ethanol and washed with ethanol. The precipitate was dissolved in 0.005 M citrate - 0.1 M NaCl and used as an antigen after adjusting the pH to 7.0 with NaHCO₃.

**Protein Determination**

Protein was determined by the Lowry method (1951) using bovine serum albumin (Armour) as a standard.

**RIA and DIA Determinations**

RIA was determined colorimetrically by the Orcinol method (Schenider, 1955) using soluble liver RIA (MCC) as the standard. DIA was determined by the diphenylamine reaction (Dische, 1955) using calf thymus DIA (MCC) as the standard.

**Spectrophotometric Characterization of Antigens**

Ultraviolet spectrophotometry was used to study the absorption spectra of the various antigen fractions, using a Beckman model DU spectrophotometer.

**Denaturation of DIA**

This was done according to the method suggested by Reiser (1964). One mg/ml of a DIA solution in 0.015 M NaCl and 0.0015 M sodium citrate was denatured by heating at 100°C for 10 minutes in presence of 1% formaldehyde in a boiling water bath, followed by rapid chilling at 0°C.
Determination of the Total Protein in Subcellular Particles

Intact particles (nuclei, ribosomes, mitochondria) were washed with 5% TCA and then with 95% cold ethanol. The protein was freed from the nucleic acid by hydrolysis in 0.3 M KCl for 12 hours at 37°C. All remaining protein was reprecipitated with 5% TCA. Both aliquots of the precipitates were combined and the protein concentration was determined by the Lowry method as described above.

Immunological Immunization

Forty-four female albino rabbits were injected by way of the foot pads or intramuscularly with different fractions obtained from both normal and malignant tissues incorporated in complete Freund's adjuvant. The initial dose was 10-12 mg of total protein. A booster injection was given 2 to 3 weeks later. Each rabbit received a total of approximately 15-20 mg of protein except for rabbits immunized with nuclei. Some of these were given a series of booster injections and received as much as 50-60 mg of protein. The rabbits were bled 6 to 8 days after the last injection. Individual sera were separated and stored at 0°C. The sera were not pooled and each serum was tested separately.

Prior to the injections of antigens, 10 cc of blood was drawn from each rabbit from which a base line serum was obtained. Six rabbits were given only Freund's adjuvant as controls.
Hemagglutination

Hemagglutination reactions were carried out by the technique described by Eigley et al. (1963) in which antigens were covalently coupled to human group 0 Rh(+) erythrocytes with bis-diazotized benzidine. A stock solution of EDB was made according to the procedure described by Gordon et al. (1958). To 0.23 g of benzidine in 45 ml of 0.2 M HCl was added 0.175 g of NaN₂ in 5.0 ml of distilled water at 0°C. The reaction was permitted to proceed for 30 minutes with intermittent stirring. The material was quickly frozen in 2.0 ml aliquots at -78°C, and stored at -25°C. The EDB was taken out of the freezer only at the time of coupling and a 1:16 dilution in phosphate buffer pH 7.3 was used for coupling.

Freshly obtained human group 0 Rh(0) cells were washed 3 times with 0.15 M phosphate buffer, pH 7.3. A two percent solution in phosphate buffer was then made of the washed packed erythrocytes. The concentrations of 1 mg/ml of protein of the various fractions were used for coupling. DNA (native as well as denatured), nucleotides, nucleosides, and bases were used 1000 ug/ml in phosphate buffer for coupling to erythrocytes.

The antigens were added to the erythrocyte suspension in phosphate buffer and five-tenths millimeter of 1:16 EDB was added to each reaction tube and the tubes were incubated for 10 minutes at room temperature with frequent shaking. The cells were washed once with a 1:100 normal rabbit serum-saline mixture. The normal rabbit serum was previously inactivated at 56°C for 30 minutes and absorbed
with human group 0 Rh(+) cells. A 2% suspension of the washed cells
was made in the 1:100 rabbit serum-saline solution. One tenth milli­
liter quantities of the cell suspension were added to 0.5 ml of
various dilutions of the inactivated test antisera and the tubes
were incubated for 30 minutes at 37°C after which they were centri­
fuged for 30 seconds in the Adam's Serofuge and observed for aggluti­
nation by gently disturbing the sedimented cells.

Stock solutions of calf thymus DNA (mDC), soluble liver RNA (mEC),
nucleosides, nucleotides, and bases (obtained from mEC) were prepared
in phosphate buffer 0.15 M pH 7.3, at a concentration of 1 mg/ml.
Polyctydylc acid (Molecular weight 2.5 x 10^4), polyuridylic acid
(m. wt. 50,000) and polyadenylic acid (m. wt. 50,000) were obtained
from Cal Biochemical Corporation and stock solutions were made as
above. These solutions were kept at 4°C and thawed only at the time
of use.

Hemagglutination inhibition experiments were done as described
by Bigley et al. (1963). Sera and inhibitors (50-1000 µg) were
usually incubated at room temperature as well as 37°C for 60 minutes
before adding the coupled red cells. The tubes were incubated 20 to
60 minutes at 37°C, centrifuged and observed for agglutination.

Absorption of Sera

Prior to absorption all sera were inactivated at 56°C for 30
minutes. Absorptions with different erythrocytes were performed as
follows:
Rabbit erythrocytes were pooled from citrated rabbit blood. Two parts serum were mixed with 1 part of packed cells and kept at room temperature for 30 minutes. The suspension was centrifuged and the serum collected. This procedure was repeated a second time and was followed by two more absorptions at 37°C for 30 minutes.

Absorptions with human red cells were carried out in a similar manner using the same proportions of Groups A+, B+, AB+, and O+ cells and serum. The cells were sedimented after centrifugation at 4000 rpm and the serum was collected.

Absorption with Tissue Homogenates

In order to eliminate tissue specific antigens, sera were absorbed with various human organs. The organs used were noncancerous normal liver, kidney, spleen, colon and lung obtained from human cadavers. Organs were cut in small pieces with stainless steel scissors and placed in 0.25 M sucrose phosphate buffer, pH 7.3, 0.15 M. The suspension was then homogenized in a high speed Waring blender equipped with stainless steel blades for 5 minutes. The homogenate was then washed 2 times with sucrose buffer by centrifugation at 12,000 rpm in a refrigerated Sorvall centrifuge. All homogenates were pooled. Two parts of pooled homogenate were mixed with one part of inactivated serum and incubated at room temperature for 30 minutes. The suspension was then centrifuged at 12,000 rpm at 4°C. The absorption was repeated at 37°C and the serum collected after centrifugation.
Absorption of Antisera with Normal and Malignant Nuclei and Nuclear Fractions

Anti-normal and anti-malignant nuclear sera were absorbed with both homologous nuclei and heterologous nuclei. Two parts of nuclei were mixed with one part of antisemum, incubated at room temperature for 30 minutes, and the antisemum separated by centrifugation at 4000 x g for 20 minutes. The absorption was repeated at 37°C.

Various nuclear subfractions were coupled to human blood group O Rh(+) erythrocytes by EDE and used for absorption of antisera. Twenty-five hundredths ml of washed packed erythrocytes were coupled with various subfractions of either normal or malignant nuclei using 1.5 mg of protein and 0.5 ml of 1:16 EDE in phosphate buffer solution at pH 7.3. After incubation, the cells were centrifuged and washed with normal rabbit serum buffer solution. The sensitized erythrocytes were pooled to make 1 ml of packed cells. They were then mixed with 1 ml of inactivated antisemum and incubated at room temperature for 30 minutes. The absorption was repeated one more time at 37°C. The antisemum was collected after centrifugation. Generally, 12 mg protein of each subfraction were used to absorb 1 ml of antisemum. The absorptions were done both undiluted and 1:16 to 1:32 diluted antisera.

DNA, RNA and various nucleotides and nucleosides were coupled similarly with erythrocytes by EDE and used for absorption.
Preparation of Globulin

Globulin was prepared from absorbed antisera according to the method of Kendall (1937). Five ml of saturated \((\text{NH}_4)_2\text{SO}_4\) at room temperature were added to 10 ml of undiluted antiserum. The precipitate was centrifuged at 4000 x G in a refrigerated Sorvall centrifuge at 4°C. The precipitate was redissolved in 5 ml of distilled water and 2.5 ml of saturated \((\text{NH}_4)_2\text{SO}_4\) solution was added slowly with stirring. The precipitate was collected by centrifugation, redissolved in distilled water, and dialyzed 4°C against frequent changes of distilled water for 24 hours. The dialysis was then repeated against phosphate buffer saline pH 7.3, 0.015 M NaCl.

Gel Diffusion Analysis

Gel diffusion method of Ouchterlony (1964) was used to study the reactions between nonspecific antinuclear globulin and DNA-P. A 0.1% solution of ion agar (Consolidated Laboratories) was made in 0.1 M glycine solution containing 1:10,000 merthiolate and poured into plates. When the agar had solidified the plates were placed into veronal buffer pH 8.2, 0.005 M, and the buffer allowed to diffuse into the gel for 2 to 3 hours, giving the gel the correct pH and ionic strength. Before use, the plates were maintained at 5°C for 24 hours.

Ammonium sulfate precipitated globulin was dialyzed against frequent changes of distilled water for 24 hours and then against 0.1 M glycine solution. Two hundred fifty ml of 0.1 M glycine solution were used to dialyze 5 cc of globulin, which was examined for
precipitation with glycine extracted DIA-P in gel diffusion tests. The plates were incubated at 4°C and then at 27°C and the precipitin lines developed within 24-72 hours. Nuclear fractions I and II (0.14 M NaCl and Tris 7.6) were examined by gel diffusion similarly with the exception that 0.16 M Phosphate buffer saline pH 7.3 was used to equilibrate the agar gel.

Spectrochotometric Method of Determining Reactions Between Purified DIA-P and Monospecific Globulin

The property of DIA-P to absorb maximally at 260 nm was utilized to study the reaction between DIA-P and monospecific globulin from anti-normal and anti-malignant nuclear sera. After a number of trials, the following standard procedure was employed. To a series of tubes each containing equal amounts (usually 80 ug/ml) of DIA-P giving an O.D. of 1.0 at 260 nm, increasing concentrations of monospecific globulin were added. The DIA-P had been dissolved in 0.1 M glycine and the globulin was dissolved in 0.007 M phosphate buffer pH 7.2. The addition of globulin was made in such a way that the final volume obtained in each case was constant. All tubes were incubated for 60 minutes at 4°C and then centrifuged at 3000 rpm in a refrigerated Sorvall centrifuge (SW-28 rotor). The ultraviolet absorption was determined before and after centrifugation. A variation of this procedure was used in which the amount of globulin was kept constant and the amount of DIA-P varied. This procedure was also utilized with
fractions of serum obtained after filtration through Sephadex G-100 and G-200.

The reaction between DNA isolated from glycine extracted DNA-P from normal and malignant nuclei with globulin from antisera to normal and malignant nuclei was also examined by the same methods.

Characterization of Antibodies

A number of methods were employed to characterize the antibodies in monospecific serum against DNA-P.

Sephadex Gel Filtration

The cross-linked dextran gel Sephadex G-100 and G-200 (Pharmacia, N.J.) were used to fractionate the sera. The gels were allowed to swell in excess of 0.1 M Tris-HCl pH 8.0 in 1 M NaCl buffer (Fahey et al., 1963) for 72 hours. A small amount of very fine particles, giving the supernatant fluid a turbid appearance, was removed by decantation. Two columns of 45 cm length and 2.5 cm diameter (Pharmacia) were used. The columns were placed in a vertical position before packing, a small amount of buffer added, and the outlet closed. Then the slurry of gel (G-200 or G-100) was added and the outlet opened. More slurry was added at frequent intervals. When the layer of packed gel reached a level about 10 cm below the top of the column, the extension tube was removed and a buffer reservoir connected. A column containing G-100 gel was connected to a column containing G-200 so that the former constituted a descending filtration and the latter, an ascending one. Before applying serum samples,
the columns were equilibrated with Tris-NaCl buffer containing a 1:10,000 dilution of merthiolate. Two and a half to three ml of whole serum was applied from the top, allowed to absorb in the gel bed and then buffer added. The flow rate of elution was kept at 20-25 ml/hour. The elution occurred without application of any pressure on the column. The effluent was passed through a Uvicord-Scanner (LKB-Produktor, Stockholm, Sweden) which absorbs at 294 nm. Fractions were collected at room temperature (25°C). Fractions I and II thus obtained were extensively dialyzed against distilled water at 4°C and then evaporated at 20°C. They were resuspended in 0.07 M phosphate buffer saline pH 7.3 and again passed through a regenerated column for further purification. These purified fractions were used in tests for antibody activity.

Treatment with 2-mercaptoethanol

A 1:6.5 to 1:7 dilution of antisera (monospecific) was treated with 0.1 M 2-mercaptoethanol (in phosphate buffer 7.3) and incubated 30 minutes at 37°C. Titration of antibody activity was immediately done without the removal of 2 ME (Uhr, et al., 1962).

Sucrose Gradient Centrifugation

A linear sucrose gradient from 5 to 25 percent (V/V) sucrose in 0.05 M Tris pH 7.4 was prepared according to Uhr et al. (1962). One ml of monospecific antisera, diluted to 1:5 in the same buffer, was layered on the surface. The tubes were centrifuged at 35,000 rpm in a Beckmen Spinco Model L-2 centrifuge equipped with a SW39
rotor for 24 hours. The cellulose nitrate tubes were punctured at the bottom and 6 to 3 drops of fractions were collected from the bottom. The protein concentration was determined from spectrophotometric examination at 200 nm (Beckman DU). The globulin distribution was tested by hemagglutination.

Analytical Ultracentrifugation of Globulin Fractions of Non-specific Serum by Sephadex Filtration

Ultracentrifugation was performed in Model 3 Spinco ultracentrifuge using an An-D rotor with single sector cells. The centrifugation was carried out at 50,000 rpm at approximately 20°C. The samples were dissolved in 0.1 M Tris pH 7.3 in concentrations of 2.5 to 5 mg/ml protein. Photographs were taken at various intervals after top speed was reached.
CHAPTER III

Experimental Results

As mentioned in the Materials and Methods section, forty-four female albino rabbits were injected initially into the footpads with subcellular fractions obtained from normal and malignant tissues, incorporated in complete Freund's adjuvant. Subsequent booster doses were given intramuscularly. The sera obtained from each individual rabbits were tested for hemagglutinins with homologous antigen. As shown in Table I, good antibody response was obtained with all fractions. The data shown in Table I are representative of the response in all animals. However, in all sera tested, antibodies to normal human O red cells were consistently present. Since the antigen carrier in all tests was group O human erythrocytes, the specificity of the reactions was questionable. In case of animals given adjuvant alone no significant response was obtained. Neither did the control sera, obtained from the animals before immunization, show antibody activity with the various tissue fractions.

The fact that immunization of rabbits with cancerous and non-cancerous tissue fractions resulted in the production of antisera having a mixture of antibodies to homologous antigens and group O human red blood cells as shown by hemagglutination, was expected, since the antigens involved in tissues are heterogeneous in character and of a very complex nature. It was, therefore, significant to examine whether the antibodies to group O red blood cells, which were consistently present in high titer in the sera from animals immunized
<table>
<thead>
<tr>
<th>Test Serum</th>
<th>Test Fraction</th>
<th>Reciprocal of Serum Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti- N. nuclei</td>
<td>N. Nuclei</td>
<td>1024</td>
</tr>
<tr>
<td></td>
<td>M. Nuclei</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td>MHC+</td>
<td>16</td>
</tr>
<tr>
<td>Anti- M. nuclei</td>
<td>N. Nuclei</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>M. Nuclei</td>
<td>1024</td>
</tr>
<tr>
<td></td>
<td>MHC+</td>
<td>1024</td>
</tr>
<tr>
<td>Anti- M. mitochondria</td>
<td>N. Mitochondria</td>
<td>1024</td>
</tr>
<tr>
<td></td>
<td>M. Mitochondria</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td>MHC+</td>
<td>4</td>
</tr>
<tr>
<td>Anti- M. mitochondria</td>
<td>N. Mitochondria</td>
<td>1024</td>
</tr>
<tr>
<td></td>
<td>M. Mitochondria</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td>MHC+</td>
<td>512</td>
</tr>
<tr>
<td>Anti- M. ribosome</td>
<td>N. Ribosome</td>
<td>1024</td>
</tr>
<tr>
<td></td>
<td>M. Ribosome</td>
<td>1024</td>
</tr>
<tr>
<td></td>
<td>MHC+</td>
<td>32</td>
</tr>
<tr>
<td>Anti- M. ribosome</td>
<td>N. Ribosome</td>
<td>1024</td>
</tr>
<tr>
<td></td>
<td>M. Ribosome</td>
<td>1024</td>
</tr>
<tr>
<td></td>
<td>MHC+</td>
<td>1024</td>
</tr>
<tr>
<td>Adjuvant Control</td>
<td>All fractions</td>
<td>3</td>
</tr>
</tbody>
</table>

Legend:
N = Normal
M = Malignant
MHC+ = Normal Human group O+ blood cells
with tissue fractions, could be removed by absorption with red blood cells. The data in Table II show that absorption with rabbit erythrocytes, human blood group 0 positive cells, as well as A, B and AB cells failed to remove anti-red blood cell antibodies from antisera to normal and malignant nuclei.

Thus, the question of the specificity of the reaction still remained unresolved. It seemed, therefore, logical to assume that some common antigenic determinant was present in both nuclear fractions and human red blood cells. This assumption was in part strengthened by the result obtained by the subsequent absorption of homologous antisera with whole nuclear fractions. Data in Table III summarize the results of several such experiments. It can be readily seen that when normal as well as malignant nuclei were used to absorb their homologous antisera, practically all demonstrable antibodies were removed. However, when cross absorptions were tried, titers of antibodies to red cells were reduced in anti-malignant sera and practically abolished from anti-normal sera. Antibodies to both types of nuclei were still demonstrable in both antisera. These experiments indicated that in addition to antibodies to red cells, there were antibodies distinctly specific for nuclear antigens and there appeared to be some antigenic differences between normal and malignant nuclear fractions.

It is well known that immunization with complex antigenic entities gives rise to not only antibodies against the whole molecule but also against smaller degraded products. Therefore, the nuclei were sub-fractionated and the various products tested on antigens with the anti-nuclear sera. As shown in Figure 2, Fractions soluble in saline and
## TABLE II

ABSORPTION OF ANTI-NUCLEAR SERA WITH RABBIT AND HUMAN ERYTHROCYTES

<table>
<thead>
<tr>
<th>Absorption with</th>
<th>Test Serum</th>
<th>Test Antigens</th>
<th>Reciprocal of Serum Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>Anti-N. Nuclei</td>
<td>N. Nuclei</td>
<td>1024</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MIO+</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Anti-N. Nuclei</td>
<td>N. Nuclei</td>
<td>1024</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MIO+</td>
<td>1024</td>
</tr>
<tr>
<td>4 x with</td>
<td>Anti-N. Nuclei</td>
<td>N. Nuclei</td>
<td>1024</td>
</tr>
<tr>
<td>Rabbit Erythrocytes</td>
<td></td>
<td>MIO+</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Anti-N. Nuclei</td>
<td>N. Nuclei</td>
<td>1024</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MIO+</td>
<td>1024</td>
</tr>
<tr>
<td>4 x with</td>
<td>Anti-N. Nuclei</td>
<td>N. Nuclei</td>
<td>1024</td>
</tr>
<tr>
<td>RBC+</td>
<td></td>
<td>MIO+</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Anti-N. Nuclei</td>
<td>N. Nuclei</td>
<td>1024</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MIO+</td>
<td>1024</td>
</tr>
<tr>
<td>4 x with</td>
<td>Anti-N. Nuclei</td>
<td>N. Nuclei</td>
<td>1024</td>
</tr>
<tr>
<td>A, B, AB Erythrocytes</td>
<td></td>
<td>MIO+</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Anti-N. Nuclei</td>
<td>N. Nuclei</td>
<td>1024</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MIO+</td>
<td>512</td>
</tr>
</tbody>
</table>

**Legend:**

- N = Normal
- M = Malignant
- MIO+ = Normal Human group C+ Erythrocytes
### TABLE III

**ABSORPTION OF ANTI-NUCLEAR SERA WITH NORMAL AND MALIGNANT NUCLEI**

<table>
<thead>
<tr>
<th>Absorption with</th>
<th>Anti-Serum</th>
<th>Antigen</th>
<th>Reciprocal of Serum Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>$4\times$ N. nuclei</td>
<td>Anti-normal nuclei</td>
<td>N. nuclei</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Anti-normal nuclei</td>
<td>M. nuclei</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Anti-normal nuclei</td>
<td>M. nuclei</td>
<td>-</td>
</tr>
<tr>
<td>$4\times$ M. nuclei</td>
<td>Anti-normal nuclei</td>
<td>N. nuclei</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Anti-normal nuclei</td>
<td>M. nuclei</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Anti-normal nuclei</td>
<td>M. nuclei</td>
<td>2</td>
</tr>
<tr>
<td>$4\times$ M. nuclei</td>
<td>Anti-IT, nuclei</td>
<td>N. nuclei</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>Anti-IT, nuclei</td>
<td>M. nuclei</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Anti-IT, nuclei</td>
<td>M. nuclei</td>
<td>2</td>
</tr>
<tr>
<td>$4\times$ M. nuclei</td>
<td>Anti-normal nuclei</td>
<td>N. nuclei</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Anti-normal nuclei</td>
<td>M. nuclei</td>
<td>256</td>
</tr>
<tr>
<td></td>
<td>Anti-normal nuclei</td>
<td>M. nuclei</td>
<td>64</td>
</tr>
</tbody>
</table>

N=Normal; M=Malignant; NHC+=Normal Human group C+

### TABLE IV

**MEAN CONCENTRATION OF RIA, DNA AND PROTEIN IN VARIOUS NUCLEAR SUBFRACTIONS:**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>RIA mg/ml</th>
<th>DNA mg/ml</th>
<th>Protein mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1.2</td>
<td>1.13</td>
<td>5.2</td>
</tr>
<tr>
<td>II</td>
<td>4.95</td>
<td>1.4</td>
<td>4.6</td>
</tr>
<tr>
<td>III</td>
<td>0.12</td>
<td>3.3</td>
<td>6.8</td>
</tr>
<tr>
<td>Histone</td>
<td>-</td>
<td>-</td>
<td>2.6</td>
</tr>
</tbody>
</table>

53
Tris buffer, as well as deoxyribonucleoprotein and histone were isolated from both normal and malignant nuclei. Treatment of the isolated nuclei with 0.14 M NaCl and 0.1 Tris buffer pH 7.6 was employed to remove various ribonucleoprotein particles and other soluble proteins. Table IV shows the mean concentrations of protein, ribonucleoprotein and deoxyribonucleoprotein in the four fractions. It can be seen from the data that each subfraction contained protein, RNA and DNA with the exception of the histone fraction which as expected was predominantly protein. Traces of RNA were also detected in the deoxyribonucleoprotein fraction which was composed of 67 per cent protein and 32 per cent DNA. It is evident that it is difficult to obtain essentially pure fractions from the fractionation procedure described.

The subfractions thus obtained were coupled to Group C human red blood cells by DDB and tested for their antigenic activity by hemagglutination in homologous antisera. Antibodies were found for each of the four fractions as seen in Table V, but the highest titers were demonstrable with Fraction III. However, normal human O red cells also reacted. Hence, both anti-normal and anti-nuclear sera were absorbed twice with each fraction coupled to Group C human red cells. The data in Table V shows that absorption with the heterologous fractions completely removed all antibodies against the group O erythrocytes leaving antibodies in various titers specific for the nuclear fractions and independent of the antibody specific for the carrier red cells.

In view of the specific differences among the fractions and particularly the absorbed anti-malignant sera for malignant nuclear
subfraction III, subsequent experiments were designed to examine these differences in greater detail. Globulins were separated from monospecific anti-malignant nuclear sera by ammonium sulfate precipitation (fifty per cent saturation at room temperature). They were redissolved in distilled water and dialyzed against frequent changes of distilled water to remove ammonium and sulfate ions and finally against buffer pH 7.3 as described in the Materials and Methods section. These were then successively absorbed two times with each normal and malignant nuclear subfraction coupled to Group O erythrocytes with the exception of Fraction III from malignant nuclei. They were then tested for hemagglutinins. This procedure, as shown in Table VI, indicates that the remaining serum globulins were highly specific for Fraction III of malignant nuclei, i.e., monospecific globulins.

As it was pointed out in the data presented in Table IV, subfraction III consisted of DNA and protein with traces of RNA. To determine whether the specificity of the globulins was directed toward the protein or the DNA, the fraction was treated with trypsin as described in the Materials and Methods section. The treatment was allowed to proceed for one hour. The trypsin-treated antigen was precipitated with two volumes of 70 per cent ethanol and the washed precipitate, after redissolved into 0.14 M NaCl, was coupled to erythrocytes and tested for hemagglutination with the monospecific globulins. It is evident from data in Table VI that while there was not a significant decrease in the titer of the hemagglutinins for trypsin-treated antigen the amount of hemagglutination produced by each
### TABLE V

**ABSORPTION OF ANTI-NUCLEAR SERA WITH NUCLEAR SUBFRACTIONS**

<table>
<thead>
<tr>
<th>Absorption</th>
<th>Test Ag.</th>
<th>Anti-normal nuclear sera</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Reciprocal of serum dilution</td>
</tr>
<tr>
<td>2 ×</td>
<td>N. Nuclear Fraction</td>
<td></td>
</tr>
<tr>
<td>I₁⁻</td>
<td>I₁⁺</td>
<td>⅓</td>
</tr>
<tr>
<td>II⁻</td>
<td>II⁺</td>
<td>8</td>
</tr>
<tr>
<td>III⁻</td>
<td>III⁺</td>
<td>8</td>
</tr>
<tr>
<td>Histone₁⁻</td>
<td>Histone₁⁺</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>E₁⁺</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Absorption</th>
<th>Test Ag.</th>
<th>Anti-malignant nuclear sera</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Reciprocal of serum dilution</td>
</tr>
<tr>
<td>2 ×</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I₁⁻</td>
<td>I₁⁺</td>
<td>128</td>
</tr>
<tr>
<td>II⁻</td>
<td>II⁺</td>
<td>22</td>
</tr>
<tr>
<td>III⁻</td>
<td>III⁺</td>
<td>512</td>
</tr>
<tr>
<td>Histone₁⁻</td>
<td>Histone₁⁺</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>E₁⁺</td>
<td>-</td>
</tr>
</tbody>
</table>

N = Normal  
M = Malignant  
E₁⁺ = Normal Human group C⁺ erythrocytes
### TABLE VI

Monospecific Anti-Malignant Serum Globulins
For Subfraction III of M. Nuclei

<table>
<thead>
<tr>
<th>Absorption*</th>
<th>Test Antigen</th>
<th>Control</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>128</th>
<th>256</th>
<th>512</th>
<th>1024</th>
</tr>
</thead>
<tbody>
<tr>
<td>I&lt;sub&gt;N&lt;/sub&gt;</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>II&lt;sub&gt;N&lt;/sub&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>III&lt;sub&gt;N&lt;/sub&gt;</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>IV Histone N</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>I&lt;sub&gt;M&lt;/sub&gt;</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>II&lt;sub&gt;M&lt;/sub&gt;</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>III&lt;sub&gt;M&lt;/sub&gt;</td>
<td>-</td>
<td>4+</td>
<td>4+</td>
<td>3+</td>
<td>2+</td>
<td>2+</td>
<td>2+</td>
<td>2+</td>
<td>2+</td>
<td>2+</td>
<td>2+</td>
<td>2+</td>
</tr>
<tr>
<td>IV Histone N</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NHO+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>III&lt;sub&gt;Treated with trypsin&lt;/sub&gt;</td>
<td>-</td>
<td>2+</td>
<td>2+</td>
<td>2+</td>
<td>2+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>III&lt;sub&gt;RNA-ase&lt;/sub&gt;</td>
<td>-</td>
<td>2+</td>
<td>2+</td>
<td>2+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>III&lt;sub&gt;Treated with DNA-ase&lt;/sub&gt;</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Anti-M globulins absorbed with all normal and malignant nuclear fractions, except Fraction III<sub>M</sub>.
dilution was less. A similar effect was obtained with RNA-ase-treated fraction III, whereas treatment with DNA-ase removed practically all of the antigenic capacity. This suggested the possible DNA nature of the antigen. These data raised the possibility that DNA from malignant nuclei was different from normal. If such were the case, the differences would possibly be in some region of the whole molecule which might not be possible to detect serologically. Since it was not possible to undertake comparative studies on polynucleotide fractions of normal and malignant DNA, attempts were made to detect quantitative differences by the use of commercially available synthetic polynucleotides. Polyadenylic acid, polycytidylic acid and polyuridylic acid were coupled to red cells and used to test globulins from anti-normal nuclei sera after absorption with all nuclear fractions except normal fraction III. Such globulins are essentially monospecific. The data in Table VII indicate that a distinct specificity for any of the test antigens was not demonstrable, but that anti-malignant globulins specific for malignant fraction III possibly were highly specific for polyadenylic acid. In as much as distinct antigenic differences could be detected in nuclear fractions from normal and malignant tissue by hemagglutination, other methods were employed to substantiate these findings. Among the methods tried, was the immunodiffusion technique of Ouchterlony in which antigens and antibodies are allowed to diffuse through agar under appropriate conditions and observed for precipitation in the region of optimal concentrations. Komgold (1957) has made extensive use of this method in studies of tumor antigens. The value of the technique is its ability to separate the reactions of mixed antigens and antibodies,
<table>
<thead>
<tr>
<th>Absorption</th>
<th>Test Ag.</th>
<th>Anti-normal nuclei Reciprocal of dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>All N nuclear fraction except N III</td>
<td>III&lt;sub&gt;N&lt;/sub&gt;</td>
<td>256</td>
</tr>
<tr>
<td></td>
<td>Poly A</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Poly B</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Poly C</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-malignant nuclei</td>
</tr>
<tr>
<td>All M Nuclear fraction except III &amp; all N nuclear</td>
<td>III&lt;sub&gt;M&lt;/sub&gt;</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td>Poly A</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Poly C</td>
<td>&lt;4</td>
</tr>
<tr>
<td></td>
<td>Poly U</td>
<td>4</td>
</tr>
</tbody>
</table>
and consequently detect different antigens in a given extract or fraction and which may identify the presence of an antigen in other fractions.

First attempts to detect differences with subfraction III by gel diffusion were unsuccessful. The difficulty was, in part, due to nonspecific precipitation of subfraction III in agar gel, because of the ionic environment in the gel, although nuclear subfractions I and II readily reacted with their homologous sera. In addition, the procedure used for the isolation of subfraction III usually produced a gelatinous material as a result of the relatively high concentration of salt used for extraction, and in part, due to the denaturation of DNA-P from mechanical handling. It was, therefore, essential that in order to detect different antigenic components in this fraction by gel diffusion, it should be pure, homogenous, undenatured, and well dispersed in a relatively low concentration of solvent. As pointed out in the Materials and Methods section, the extraction of whole nuclei with glycine completely fulfilled these criteria.

Figure 3 shows the absorption spectra of malignant nuclear subfraction III isolated both by 2 M NaCl and 0.1 M glycine. As can be readily seen from the figure, maximum absorption by both extracts was at 260 mu and minimum at 240 mu. However, the ratio of maximum over minimum was between 1.5 to 1.6. In addition, mention has already been made of traces of RNA found in DNA-P extracted with 2 M NaCl, whereas, RNA could not be detected by the Orcinol method in glycine extracted DNA-P. Data on the protein content by the Lowey method and
Figure 3. The ultraviolet spectra of DNA-P isolated (i) • - glycine extraction; (ii) ▲ - 2 M NaCl extraction.
DNA by the diphenylamine reaction in the glycine extract are shown in Table VIII. It is evident from the data that the fraction contained approximately 29.6 per cent DNA and 71.4 per cent protein.

Since this DNA-P fraction demonstrated maximum absorption at 260 μm, ultraviolet spectrophotometry was employed to study the reaction between glycine extracted DNA-P and homologous nonspecific globulins. Figure 4 shows the spectrum of DNA-P isolated from malignant nuclei mixed with nonspecific globulins for normal and malignant nuclei before and after centrifugation. From the spectrum it is evident that nonspecific anti-malignant-nuclei globulin readily removed DNA-P from the solution while normal DNA-P was not as effective. The converse reaction was also tried as shown in Figure 5. This shows the spectrum of DNA-P isolated from normal nuclei and the same antigen plus normal and malignant nuclear nonspecific globulin. It is interesting to note that whereas normal DNA-P reacted well with nonspecific anti-normal nuclear globulin, malignant DNA-P did not show marked reactivity with anti-normal nuclei globulin.

In another series of experiments, different amounts of nonspecific anti-normal or anti-malignant globulins were mixed with equal amounts of malignant DNA-P (optical density, 1.0 at 260 μm), in such a way as to obtain a constant volume. The ultraviolet spectrum was examined before and after centrifugation. As can be seen from Figure 6, in which malignant DNA-P was used to see reactivity with nonspecific normal and malignant nuclear globulin, Curves A and B show that malignant globulin reacted much better with malignant DNA-P than did normal globulin. The increase in optical density at
### TABLE VIII
DNA and Protein in Glycine Extracted Malignant DNA-P (fraction III)

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Protein mg/ml</th>
<th>DNA mg/ml</th>
<th>% Protein</th>
<th>% DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.60</td>
<td>0.73</td>
<td>69.0</td>
<td>31.0</td>
</tr>
<tr>
<td>2</td>
<td>1.80</td>
<td>0.568</td>
<td>75.0</td>
<td>25.0</td>
</tr>
<tr>
<td>3</td>
<td>4.21</td>
<td>1.66</td>
<td>71.7</td>
<td>28.3</td>
</tr>
<tr>
<td>4</td>
<td>3.26</td>
<td>1.37</td>
<td>70.4</td>
<td>29.6</td>
</tr>
<tr>
<td>5</td>
<td>1.54</td>
<td>0.55</td>
<td>73.0</td>
<td>27.0</td>
</tr>
<tr>
<td>6</td>
<td>2.47</td>
<td>1.00</td>
<td>71.2</td>
<td>28.8</td>
</tr>
<tr>
<td>7</td>
<td>2.10</td>
<td>0.86</td>
<td>70.1</td>
<td>30.9</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td></td>
<td><strong>71.4</strong></td>
<td><strong>29.6</strong></td>
</tr>
</tbody>
</table>
Figure 4. • - The ultraviolet spectra of glycine DNA-P from malignant nuclei (50 ug/ml), and of the supernatant fluid after addition of
(i) ▲ - anti-malignant (70 mu/ml) nuclear monospecific globulin;
(ii) ■ - anti-normal nuclear monospecific globulin (70 ug/ml)
and centrifuged.
Figure 5. (i) ■ - The ultraviolet spectra of glycine DNA-P from normal nuclei (50 ug/ml) and of the supernatant after addition of (ii) ▲ - anti-normal nuclear monospecific globulin (70 ug/ml); (iii) ○ - anti-malignant nuclear monospecific globulin (70 ug/ml) and centrifuged.
Figure 6. Optical density at 260 nm of constant amount DNA-P from malignant nuclei equated to 100% with varying concentrations of monospecific (A) anti-malignant (B) anti-normal nuclear globulins, before centrifugation and (C) anti-malignant (D) anti-normal nuclear monospecific globulin after centrifugation.
260 nm is the result of light scattering due to aggregation of DHA-P and immune globulin. The curves C and D show that after centrifugation malignant globulin was more efficient in progressively precipitating malignant DHA-P from the reaction mixture than normal globulin. The characteristic flat region shown by curve (c) in analogous to the conventional precipitation curve, in which a region of antigen excess may be expected. Figure 7 summarizes the experiments done with normal DHA-P as above with monospecific normal and malignant globulins. It is apparent from the figure that homologous immune globulins was much more effective both in formation of aggregation and precipitation of antigen than heterologous globulin. These experiments clearly point out the significant differences in the reactivity or normal DHA-P and malignant DHA-P. It should be noted that base line globulin (before immunization) did not show any significant reactivity either with normal DHA-P or malignant DHA-P and hence the reactions were undoubtedly specific.

The results of hemagglutination studies indicated the major role of DNA in the antigenic differences between normal DHA-P and malignant DHA-P. The following experiments were performed to test and confirm hemagglutination findings by ultraviolet spectrophotometric methods.

DNA was isolated from both normal DHA-P and malignant DHA-P by the methods described in the section of Materials and Methods. The preparation obtained by these methods was of fairly high quality, being free of protein after assay by the Lowry method. Figure 8
Figure 7. Optical density at 260 nm of constant amount of DNA-P from normal nuclei equated to 100% with varying concentrations of monospecific (C) anti-normal (A) anti-malignant monospecific globulin before centrifugation and (B) anti-normal (B) anti-malignant nuclear monospecific globulin after centrifugation.
illustrated that the absorption spectra of the preparation is typical of DNA with a typical absorption maximum at 260 μm and a minimum of approximately 237 μm. Experiments designed as above by using a constant amount of DNA having an optical density of 1.0 at 260 μm, and adding various concentrations of monospecific globulin, did not show any reactivity. However, DNA was denatured in the presence of one per cent formaldehyde followed by rapid chilling, and used as an antigen, relatively weak reactivity was observed. Figure 9 demonstrates the reaction of malignant denatured DNA with monospecific anti-normal and malignant nuclear globulins. It is to be noted that the marked differences in reactivity observed with DNA-P were greatly reduced both in aggregation and precipitation. Experiments with normal denatured DNA with homologous and heterologous globulins also failed to reveal significant differences as shown in Figure 10. These data, therefore, are suggestive of the fact that the DNA-P complex is involved in the differential reactivity with normal and malignant nuclear globulins.
Figure 8. The ultraviolet spectra of DNA isolated from (i) • - 2 M NaCl DNA-P; (ii) ▲ - glycine extract DNA-P.
Figure 9. Optical density at 260 μm of constant amount of denatured DNA from malignant nuclei equated to 100% with varying concentrations of monospecific (A) anti-malignant (C) anti-normal nuclear globulins before centrifugation and (B) anti-malignant (D) anti-normal monospecific globulin after centrifugation.
Figure 10. Optical density at 260 μm of constant amount of denatured DNA from normal nuclei equated to 100% with varying concentrations of monospecific (A) anti-normal (C) anti-malignant nuclear globulins before centrifugation and (B) anti-normal and (D) anti-malignant monospecific globulin after centrifugation.
Gel Diffusion Analysis of Nuclear Antigens

Since tissue and organ specific antigens are unevenly distributed in various fractions of cells, it was necessary to absorb anti-normal and malignant nuclear sera with homogenates of liver, kidney, colon, lung and erythrocytes from Groups A, B, AB and 0 in order to eliminate organ or tissue specific antibodies. Such absorbed sera were then used for antigenic analysis by gel diffusion. Figure 11 shows the reactions between anti-malignant nuclei serum (center well) and 0.14 M NaCl nuclear fraction I extracted by 0.14 M NaCl from both normal and malignant nuclei. Note that very little qualitative differences may be observed. It is seen that there are three antigens (a, b, and c) in malignant nuclear fraction I. The same antigens are also found in the normal nuclear fraction I, although the concentration of antigen b seemed to be markedly reduced.

Figure 12 depicts the results of similar experiments with subfraction II (0.1 Triton X-100). At least three precipitin lines (1, 2, 3) are apparent. Antigen 1 in the malignant fraction gave a clear precipitin line whereas 2 and 3 produced very faint lines. In contrast to this, Antigens 1 and 2 seemed to be reduced in normal nuclear subfraction II. These quantitative differences cannot be interpreted in terms of antigen deletion or gain in the cancer tissue fractions, since the tissue antigens are not only unevenly distributed but also vary in their concentration in different fractions.

In general, it is easier to detect altered protein structure or abnormal
protein synthesis than a complete lack of or quantitative differences in antigen contents by gel diffusion.

Prior to examining the specificity of monospecific sera with DNA-P (malignant subfraction III) by immunodiffusion, homologous sera absorbed with various normal organs and blood cell groups were tested for antibodies to DNA-P by hemagglutination. The results in Table IX show that agglutinins for the Group O red cell carrier were almost entirely removed, while a much higher titer of antibody for normal DNA-P remained and even more antibody to malignant DNA-P was still present.

More significant differences in the reactivity of normal DNA-P and malignant DNA-P with monospecific globulins were obtained in other gel diffusion experiments. The center well in Figure 13 contained 10 mg of anti-malignant nuclear globulins. Wells A, B, C had 1000 µg each of malignant DNA-P. Well D contained the same concentration of normal DNA-P. It is to be noted that only one precipitin line was obtained with homologous antigen and antibody and that malignant monospecific globulins did not react with normal DNA-P. This experiment was repeated using a five-fold increase in the concentration of normal DNA-P, but still no lines of precipitation were observed, indicating that these findings are not quantitative. Similarly, increased concentrations of specific globulins produced only one line or precipitation with malignant DNA-P. It is also worth noting that malignant DNA-P isolated by glycine extraction from malignant tissue of four different individuals reacted with
Figure 11. Center, anti-malignant nuclear serum; (1) and (2), nuclear subfraction I (0.14 M NaCl extract) from malignant nuclei; (3) and (4) nuclear subfraction I from normal nuclei.

Figure 12. Center, anti-malignant nuclear serum; (A) and (B), nuclear subfraction II (0.1 M Tris, pH 7.6 extract) from malignant nuclei; (C) and (D) nuclear subfraction II from normal nuclei.
**TABLE IX**

Effect of Organ Absorption on DNA-P Antibodies in Anti-Normal and Anti-Malignant Sera.

<table>
<thead>
<tr>
<th>Absorption</th>
<th>Test Antigen</th>
<th>Antiserum</th>
<th>Cell Control</th>
<th>Reciprocal of Dilution Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate of kidney, liver, colon, lung and blood groups</td>
<td>N-DNA-P</td>
<td>Anti-N</td>
<td>-</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>NHO+</td>
<td></td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>M-DNA-P</td>
<td>Anti-M</td>
<td>-</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td>NHO+</td>
<td></td>
<td>-</td>
<td>4</td>
</tr>
</tbody>
</table>

N = Normal  
M = Malignant
globulins from any one anti-malignant nuclear serum by the formation of lines of identity (Figure 14). When anti-normal nuclear monospecific globulins were tested against malignant DNA-P and normal DNA-P, the same specificity was noted. That is, normal DNA-P reacted only with anti-normal globulins, but not with malignant DNA-P (Figures 15 and 16).

**Characterization of Antibodies in Monospecific Malignant Antiserum:**

Numerous methods were tried to characterize the nature of antibody for malignant DNA-P in monospecific sera. Three separate fractions were obtained by gel filtration of the serum through columns of Sephadex G-100 (descending) and G-200 (ascending) as indicated by three separate peaks on the recording paper (Figure 17). The data in Table 1 show that hemagglutinins were present in both fractions I and II, while Fraction III contained no detectable antibody. Usually, the first fraction from such filtration contains IgM (19S) antibodies, those of fraction II are of the IgG (7S) variety. However, treatment with 2-mercaptoethanol (2-ME) which is known to reduce intramolecular disulfide bonds, reduced the hemagglutinating capacity by only 20% (Table 2).

When the samples of monospecific sera were subjected to sucrose gradient ultracentrifugation (Figure 18), considerable hemagglutination activity was found in the contents of the bottom four fractions supposedly containing macroglobulin IgM. This was further confirmed by the fact that such antibody activity was in part destroyed by treatment of these fractions with 2-ME. However, significant activity
Figure 13. Center, monospecific anti-malignant nuclear globulin; (A) (B) (C) glycine extract DNA-P from malignant nuclei of three carcinomas; (D) glycine DNA-P from normal nuclei.

Figure 14. Center, anti-malignant monospecific nuclear globulin; (1) (2) (3) (4) glycine extract DNA-P from four different carcinomas showing lines of identity.
Figure 15. Center, anti-normal nuclear monospecific globulin; (1) and (2), glycine DNA-P from normal nuclei; (3) and (4) glycine DNA-P from malignant nuclei.

Figure 16. Center, anti-normal nuclear monospecific globulin; (A) and (B), glycine DNA-P from normal nuclei of two individuals showing lines of identity.
Figure 17. Elution pattern of monospecific serum of Sephadex G-100 (descending) and G-200 (ascending) gel filtration.
Figure 16. Sarcose density gradient ultracentrifugation of nonspecific anti-malignant nuclear serum.  
- protein concentration measured by absorbance at 280 nm; bar - antibody activity for malignant DNA-P assayed by hemagglutination.
TABLE X

M-DNA-P Activity in Monospecific Serum of Fractions Obtained From Sephadex Gel Filtration

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Hemagglutination titer (reciprocal of dilution)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before 2 ME Treatment</td>
</tr>
<tr>
<td>I</td>
<td>256</td>
</tr>
<tr>
<td>II</td>
<td>128</td>
</tr>
<tr>
<td>III</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 19. Optical density at 260 μm of constant amount of DNA-P from malignant nuclei equated to 100% with varying concentrations of (A) anti-malignant serum fraction I from Sephadex; (B) fraction II of anti-malignant; (E) Sephadex fraction I and (F) fraction II of anti-normal nuclear monospecific serum before centrifugation; (C) same as A; (D) same as B; (G) same as E; and (H) same as F, after centrifugation.
Figure 20. Optical density at 260 nm of constant amount of DNA-P from normal nuclei equated to 100% with varying concentrations of (A) anti-normal serum fraction I from Sephadex; (C) fraction II of monospecific anti-normal nuclear serum (E) fraction I and (G) fraction II of monospecific malignant nuclear serum before centrifugation and (B) same as A; (C) same as D; (H) same as G; and (F) same as E, after centrifugation.
Figure 21. Ultracentrifuge pattern of purified fraction I from Sephadex centrifuged at 56,000 rev/min. Pattern after 32 minutes.

Figure 22. Ultracentrifuge pattern of purified fraction I from Sephadex centrifuged at 56,000 rev/min. Pattern after 64 minutes.
Figure 23. Ultracentrifuge pattern of purified fraction II from Sephadex centrifuged at 56,000 rev/min. Pattern after 36 minutes.

Figure 24. Ultracentrifuge pattern of purified fraction II from Sephadex centrifuged at 56,000 rev/min. Pattern after 80 minutes.
was also observed in fractions from the upper parts of tubes containing IgG globulins. It is therefore, apparent that hemagglutinins for malignant DNA-P were distributed in both molecular species of globulin.

The IgM and IgG fractions of monospecific serum obtained by gel filtration were also tested for reactivity with DNA-P by spectro-photometric methods. For this purpose, a constant amount of antigen having an optical density of 1.0 at 260 nm was mixed with various concentrations of fractions IgM and IgG. As may be noted in figures 19 and 20, differential reactivity observed in the entire globulin, as described earlier, is also seen in globulin fractions. It is further evident that Sephadex fraction II (IgG) is more avid in forming precipitates than fraction I (IgM).

The purity of the fractions obtained by gel filtration was examined by use of the analytical ultracentrifuge. The results are shown in Figures 21 through 24, indicating that three times purified fraction I (IgM) is quite homogeneous while fraction II (IgG) seem to be contaminated with a fast sedimenting (Possibly 19S) molecular species of globulin.

The significance and a critical evaluation of the results described in these pages will be thoroughly discussed in the next section of this dissertation.
CHAPTER IV

DISCUSSION

The aim of this research was to examine antigenic differences between normal and malignant human tissues. Much of the previous work done in this area has been with tumor extracts and antisera obtained against such extracts which were absorbed with normal tissues to remove antinormal components of the antiserum. Any activity left in such absorbed sera were considered to be due to tumor specific antigens. However, in various interpretations of the results of absorption experiments, little consideration has been given to the possibility that tumor specific antibodies may have been removed or inactivated by normal tissue components similar to, but not identical with, the tumor antigens which initially stimulated antibody production. Another aspect of the use of tumor extracts, as such, is the possibility that during their preparation, there may be partial or complete destruction, or masking of antigens. But perhaps the greatest reason giving rise to the misinterpretation of results has been the failure to use the same individual as the source of normal and tumor material. Usually, the source of normal tissues in such studies has either been noncancerous individuals or from cadavers free of malignant neoplastic disease. In addition to the fact that the normal individual's isoantigens may have been diluted by the use of a tissue pool, it is quite possible that the source of the antigens labeled as tumor specific may have been, in reality, simply due to individual antigenic differences between donors. In the
present study various cellular organelles were carefully isolated from human cancer tissue and normal tissue from the same human donors by refined biochemical techniques. Those were used for immunization of rabbits and the resultant antisera examined for specific antibodies. The immunologic comparison of normal and malignant tissue antigens was then done on each serum with its homologous antigens rather than on pooled antisera.

Interestingly, the organelles, namely nuclei, mitochondria and ribosomes, stimulated good antibody responses. Because of the role of the nucleus, especially deoxyribonucleoprotein, in gene regulation and cellular differentiation, it was of importance to examine in detail the antigenic composition of nuclear antigens from normal and malignant tissues and thus the work was particularly directed towards detecting antigenic differences in the nucleic acids, their associated proteins, and their constituents.

The data showed that in all sera tested antibodies to normal human C red cells were consistently present, regardless of the organelle antigen. They were present in relatively high concentration in antisera to fractions derived from malignant tissues. Since hemagglutination was chosen to examine the sera because of its sensitivity, it was, therefore, necessary to remove these antibodies by absorption from anti-nuclear sera in order that the specificity of the nuclear antigens could be ascertained. However, attempts to absorb out these antibodies with rabbit or human red blood cells (A, B, AB, O) were unsuccessful. The reason for this is not yet clear.
but others in this laboratory have found that these antibodies may be absorbed readily from certain antisera to other subcellular components, for example, mitochondria and ribosomes, but absorption of other antisera to these same antigens may be difficult or unsuccessful. Nevertheless, it could be safely assumed that there must be common antigenic components shared by human group O cells and nuclear antigens, since no red cell material could be found in the immunizing preparations. This assumption was particularly strengthened when absorptions with whole nuclei were consistently successful in removing group O red cell antibodies from these sera. These observations were not surprising in view of previous reports on the existence of antigens common to tumors and red blood cells. Boyden and Aptekman (1953) have made similar observations with isologous tumors spontaneous or carcinogen induced in P.A. or Lewis inbred strains of rats. They found this cross-reaction between tumors and human erythrocytes was due to a common antigen in both tumor and erythrocyte which was not related to human red cell A, B, or C substances (Aptekman and Boyden, 1954). In fact, Green (1957) has already isolated a lipoprotein fraction from rat sarcomas, carcinomas and from various human tumor extracts which has strong hemolytic and hemagglutinating activity for human erythrocytes. On the basis of extensive studies on the life span of erythrocytes in tumor-bearing hosts, he further emphasizes that one of the main causes of anemia accompanying cancer is due to the production of such antibody by hosts in response to tumor antigens which cross react with red cells.
When absorptions of hyperimmune sera with subfractions of nuclei were tried, it was noted that subfraction I and II removed these red cell antibodies leaving at the same time antibodies specific for fraction III. Since these fractions contain mainly protein and RNA with only traces of DNA, it could be that there is a cross reacting nucleotide sequence in the stroma of red blood cell and nuclear fractions. This is not surprising in view of the fact that red blood cell stroma contain 125.4 μ moles/100 ml of nucleotides and 125.5 μg of ribonucleic acid/100 ml of cells (Pennell, 1964). Preliminary attempts were made to absorb antibodies for normal human group C cells with various combinations of nucleotides, and it was found that adenyllic acid, cytidylic acid, uridine and UDP were all effective. However, the exact nature of this cross reaction should await further work before a definite conclusion can be reached.

There is, however, another possibility which cannot be overlooked in any discussion of antibodies to normal human C red cells in antisera to tumors. Since the antigenic differences between rabbit and man are so great, these antibodies might represent species specific antibodies since they showed no specificity for blood group antigens. This possibility is in part strengthened by the fact that absorptions with homogenates of human organs in conjunction with blood group substances practically removed all normal human C antibodies, while antibodies for DNA-P remained in the sera. It has been reported by Billinghan, Brent and Medawar (1956) that the histocompatibility antigens in mice reside wholly in the nuclei of the cells, and are well developed before birth. They are inactivated by lyophilization,
freezing, thawing or heating to 48.5°C. In view of the uneven distribution of histocompatibility antigens in normal and neoplastic tissues arising from different antigens present in tumors, it would not be surprising to detect cross reacting antihuman antigens in nuclear fractions. It should, however, be emphasized here that the nuclear preparations obtained by the fractionation procedure employed were free from any red blood cell material.

A second group of antibodies, also without specificity for cancer DNA-P, was absorbed by homogenates of normal organs and nuclear fractions from normal tissue. These might well be organ specific antibodies. However, it is a well known immunologic phenomenon that immunization with complex antigens not only produces antibodies to the whole molecule but also to its degradation products. This was clearly demonstrated in anti-nuclear sera, all of which contained antibodies to saline soluble, Tris-soluble proteins as well as to histone and nucleoprotein, although antibodies to the latter were consistently in higher titers. Cross absorptions with individual fractions (Table V) did reveal some quantitative differences among these fractions.

A more striking serologic specificity for DNA-P isolated from malignant nuclei was noted in Table VI, in which anti-malignant nuclear fractions, except for fraction III of malignant nuclei, still contained antibody for the latter. The possibility that this specificity was associated with DNA was shown by the fact that
treatment of the antigen with trypsin or RNA-ase did not reduce reactivity significantly, but it was markedly reduced by treatment with DNA-ase. Since trypsin treatment probably would not remove all the protein associated with DNA, the possibility of the whole DNA-P complex acting as antigen cannot completely be eliminated. Furthermore, it has been shown by various workers that antibodies to deoxyribonucleoprotein cross react with DNA from various sources.

Greenberg and Uhr (1967) have shown that in myeloma tumors which elaborate specific globulins, there are marked differences in DNA from malignant tissues and normal tissues from the same inbred strain of mice. These differences as detected by RNA-DNA hybridization experiments are in a small segment of DNA. Similar conclusions have been drawn by Sachs (1964) from work on hybridization experiments with normal tissue and polyoma induced tumor in which he has shown that there were not more than five copies of viral genome per tumor cell. These findings suggest that there are differences in small segments of DNA of normal and malignant tissue.

In view of these findings it was of interest to examine the differences at the nucleotide level by the use of monospecific globulin to normal and malignant nuclei. Since DNA antibodies are highly cross reactive, significant differences could not be detected with synthetic polynucleotides obtained commercially (Table VI). It has been shown that antibodies induced by heat-denatured DNA of one source not only cross react with heterologous DNA but also with RNA, and conversely, antibodies to RNA cross react with DNA (Flescia et al., 1964, 1965). It is also a well known fact that the specificity of
antibodies formed depends upon the composition of the antigen or hapten in the immunologic complex. Native DNA contains four nucleotides in common (thymidylate, cytidylate, adenylylate, guanylylate) and thereby may stimulate the induction of antibodies specific for each of the nucleotides alone as well as in sequences of undetermined length. However, this problem of the heterogeneity of the antibody population does not apply to antisera produced against conjugates with BSA or synthetic polypeptides containing derivatives of purines and pyrimidines. Antibodies in such sera are highly specific for the base used (Sela et al., 1965). Clearly, therefore, if antibodies to nucleic acid are to be useful in differentiating the structural features of nucleic acid from normal and malignant tissues, it will be necessary to define the heterogeneity of nucleic acid specific antibodies and thereby clearly delineate their specificity. In order to accomplish this and reduce the cross reactivity, oligonucleotides of known size and composition must be isolated from normal and malignant DNA, and coupled to different carriers. In this manner, it would be possible to determine whether or not the antibodies formed are strictly specific for the entire sequence of nucleotides. This objective could not be achieved in this study.

The reactions of the various nuclear fractions were also examined for their reactivity with anti-malignant nuclear sera by the Ouchterlony's immunodiffusion technique. The data presented on the reactions of nuclear fractions I and II from normal and malignant nuclei with
anti-malignant nuclear sera showed that there were no distinguishable qualitative differences. However, at least two antigens in normal nuclear fraction I and one in normal nuclear fraction II seemed to be present in less quantity than in malignant nuclear fractions. Because of the uneven distribution of tissue antigens in different fractions both in concentration and number, it is difficult to ascertain the significance of these quantitative differences. The same difficulty has been pointed out by Komgold (1957) who did extensive work on tissue antigens both in normal and tumorous tissue. He concluded that, in general, it was easier to show altered protein structure or abnormal protein synthesis, than the complete lack of antigen by the double diffusion gel technique.

Initial attempts to obtain reactions between monospecific globulin for Subfraction III from malignant nuclei with its homologous antigen were unsuccessful. This was in part due to the gelatinous nature of the preparation obtained with 2 M NaCl extract which did not migrate readily in the agar gel. Zubay and Doty (1959) have shown that this gelatinous condition is due to the relatively high concentration of extracted material and denaturation due to mechanical handling of the source material during the extraction. It was, therefore, necessary to obtain a pure, undenatured and well-dispersed DNA-P. As mentioned in the previous section, this was accomplished by glycine extraction. Data have been presented showing that both 2 M NaCl and glycine extracts contained traces of RNA, no RNA was detected in glycine extracts. In addition, glycine extraction does not involve extensive manipulation
of material to be extracted and hence yielded mostly undegraded and well dispersed material. To obtain specific precipitation by immuno-diffusion, it was also necessary to control rigidly the ionic environment in which the reactions were carried out, since slight changes in the salt concentration precipitates DNA-P from glycine extracts. Indeed, it was found that DNA-P was precipitated by merely mixing DNA-P with undialyzed sera - both immune and normal, and in agar gel diffusion tests with undialyzed sera, there was little, if any migration of DNA-P, but instead, a typical salt precipitation occurred. Because of these results, the salt concentration was maintained at 0.005 M, which is well below the ionic concentration producing precipitation or structural changes of DNA-P. Under these conditions, the specific reactivity of DNA-P became evident.

It is evident from gel diffusion experiments that anti-malignant nuclear monospecific globulin reacted only with malignant DNA-P and not with normal DNA-P. Conversely, anti-normal nuclei monospecific globulin reacted with only normal DNA-P. It was further evident that DNA-P isolated from all ten samples of cancerous tissue demonstrated the same specificity, as seen by the lines of identity formed from the reactions of subfractions of various tumors and the globulins. The possibility of these differences being due to organ or tissue specific antigens was eliminated by the successive absorption of the serum with homogenates of various normal organs and of blood group substances before the globulins used in the gel diffusion experiments were separated.

The above marked differential specificity of DNA-P isolated from cancerous tissue was further confirmed by the data presented on the
reactions between DNA-P antigen and homologous globulin by ultraviolet spectrophotometry. The maximum absorbance of DNA-P at 260 nm is primarily due to the DNA in the complex. This property was utilised to study the specific reaction between DNA-P and its homologous globulin. If the reaction was specific, the complex formed would be sedimented after centrifugation and consequently antigen would be removed from the solution, markedly reducing absorbance at 260 nm. The data presented showed that this was indeed the case. Only homologous antigen formed precipitates with homologous globulin. Globulin from monospecific anti-normal nuclear sera did not react with malignant DNA-P as evident from the fact that with such sera there was marked rise in absorbance at 280 nm even after centrifugation, indicating the antibody protein was in solution. In another series of experiments a constant amount of DNA-P was mixed with various concentrations of homologous globulin and incubated for an appropriate interval at 4°C. The optical density at 260 nm was taken before and after centrifugation. If the reaction was specific there would be increase in optical density at 260 nm caused by light scattering due to the formation of aggregates of DNA-P and globulin. After centrifugation, a marked decrease in optical density should occur because of the antigen being removed from solution. The results indicated a marked specificity in the reaction of malignant DNA-P with anti-malignant nuclear monospecific globulin. However, very little cross reaction occurred with anti-normal nuclear monospecific globulin. The converse was also demonstrated. It is obvious, however, from the data that anti-normal
nuclear globulin reacted minimally with malignant DNA-P as evident from the relatively small increment in optical density at 260 μm which occurred when malignant DNA-P was mixed with normal globulin. However, an examination of the curves after centrifugation showed that whereas the reaction between homologous malignant nuclear antigen and antibody produced a typical precipitation curve with a flat region in antigen excess, heterologous normal globulin not only failed to do so but was not as strongly effective in precipitating antigen. This problem of minimal cross reactivity between malignant DNA-P and anti-normal nuclear monospecific globulin can also be argued from the viewpoint of serologic specificity. The specificity of serologic reactions, although extraordinarily sharp, is still limited. Landsteiner (1945) has brilliantly defined it as "... the disproportional action of a number of similar agents on variety of related substrata." If there are few identical antigenic determinants between normal DNA-P and malignant DNA-P, as one would strongly suspect, slight cross reaction would be expected as evidenced in the above studies with spectrophotometric method. It is significant to note that native DNA isolated from both normal and malignant glycine extracted DNA-P did not react significantly with their homologous globulins. However, when denatured, in presence of formaldehyde, a weak reaction could be demonstrated. Moreover, the marked differences noted between entire malignant DNA-P and anti-normal nuclear mono-specific globulins as compared with DNA alone were considerably different. Moreover, essentially similar curves were noted for the reaction between
denatured DNA and anti-normal nuclear globulin by spectrophotometric method of reaction. As was pointed out earlier, this would be expected in view of the structural feature of DNA which contains only four common nucleotides, and hence, induces antibodies specific for each of the nucleotides of undetermined length. It would be difficult to differentiate immunologically the fine structural features of nucleic acid from normal and malignant tissue unless these heterogeneous specific antibodies are studied at the level of oligonucleotides of known composition and sequence.

The results of the present investigation strongly suggest that DNA-P from malignant nuclei is different from normal DNA-P. The differential specificity was shown by hemagglutination, gel diffusion and spectrophotometric analyses. The possibility of isoantigenic differences is most unlikely, in view of the comparison with malignant and normal tissue obtained from the same individual. It is to be emphasized that Gorer (1947) felt that isoantibodies rather than antibodies to cancer antigens appeared in animals to which tumors were inoculated. This was certainly not the case in the present study. It seems highly unlikely that all adeno carcinomas of the colon and carcinomas of the lungs share hypothetical isoantigens different from normal tissue and associated only with deoxyribonucleoprotein.

The above data are in good agreement with the findings of a number of workers. Zilber (1958) demonstrated immunologic differences between DNA-P from normal and malignant tissues by techniques of anaphylaxis and desensitization. Messineo (1961) has also reported
immunologic differences by gel diffusion analysis between DNA-P from leukemic cells and normal leukocytes. The data presented here also support the findings of Perez-Cuadrado and Haberman (1964, 1965, 1965a) who examined about one hundred specimens of human cancer immunologically and presented proof for the presence of cancer specific DNA-bound protein antigens. Similar results have been obtained by Bringhurst (1965) who isolated DNA-P's from the cells of Ehrlich ascites tumor and Sarcoma 37 which when used to immunize mice provided definite protection to challenge with the tumors, whereas DNA-P from normal cells did not produce any significant resistance.

In the present work, the molecular species of antibodies in the monospecific anti-malignant nuclear sera were separated and characterized from Sephadex filtration and sucrose gradient ultracentrifugation. The data presented showed that the hemagglutinating activity was associated to greater extent with the IgM fraction than with the IgG, although the total hemagglutinating activity was also detected in the IgG fraction. It is well known that mercaptoethanol destroyed the antibody demonstrations associated with 19S globulin but does not affect most of the 7S antibody (Bauer et al., 1961). In the results reported here, it is evident that 0.1 M mercaptoethanol, without alkylation, produced only a 20% reduction of hemagglutinin activity, indicating that antibody activity was also associated with 7S type globulin. In addition, spectrophotometric analysis showed that 7S or IgG antibody globulins were much more effective in combining with DNA-P to produce precipitation, which conforms with the generally held view that IgG antibody is a more effective precipitin.
of IgM and IgG fractions obtained from Sephadex by analytical ultracentrifugation indicated fraction I as a single peak. The second fraction demonstrated two peaks indicating possible contamination with IgM. In as much as the IgG fraction contained IgM, the possibility of a single molecular species of antibody specific for malignant DNA-P was ruled out. Treatment of pure IgM with mercaptoethanol did not destroy all antibody activity. These findings are in agreement with the results reported by various other workers. Deicher et al. (1959) have shown that DNA antibodies in the sera of individuals with systemic lupus erythematosus were mainly 7S globulin while anti-DNA-P was distributed both in 7S and 19S. In contrast, Stroller (1966) has reported that specific reactivity in rabbit antisera prepared to complexes consisting of DNA-MBSA (methylated bovine serum albumin) behaved as 19S macroglobulins as determined by Sephadex G-200 chromatography. The specific reactivity of this was directed primarily at the DNA molecule. Furthermore, this DNA-specific reactivity present in the serum was completely abolished by mercaptoethanol treatment.

In view of these findings by other describing the molecular species of immunoglobulins which contain specific DNA and DNA-P reactivities, the results of this present investigation suggest that the monospecific rabbit anti-DNA-P reactivity was contained in a heterogeneous population of immunoglobulin molecules. It has been observed that specific anti-DNA-P reactivity resides in both the IgM and IgG fractions of rabbit antisera. Furthermore, evidence of heterogeneity within an immunoglobulin class is apparent by the fact
that the DNA-P reactivity of a portion of the IgM molecules were sensitive to mercaptoethanol. This monospecific IgM fraction was characterized not only on the basis of its antigenic specificity but also by gel filtration and ultracentrifugation. It is of interest to note that the Schlieren pattern of this macroglobulin contained but one peak of protein.
CHAPTER V

SUMMARY

(1) Various cellular organelles, viz. nuclei, mitochondria and ribosomes from normal and malignant tissues from the same human donors were separated biochemically, and used to immunize forty-four rabbits. The resultant antisera were examined by hemagglutination and gel diffusion precipitation for immunologic differences between normal and malignant tissues, especially nuclei and DNA.

(2) All antisera contained antibodies to normal human group O erythrocytes which were not absorbed by A, B, O and AB human red cells, nor by rabbit erythrocytes.

(3) Absorption of antinuclear sera (both antinormal and antimalignant) with purified nuclei removed antibodies to carrier erythrocytes indicating the presence of antigenic components common to nuclei and erythrocytes. However, results of absorptions of these same sera with various organ homogenates also demonstrated the presence of species specific (anti-human) reactivities related to the human blood group substances.

(4) Antibodies to the nuclear subfractions, viz. saline soluble protein, 0.1 M Tris buffer, pH 7.6, protein, deoxyribonucleoprotein (DNA-P) and histone were also detected. Results of cross absorption.
experiments indicated a difference in reactivity of the DNA-P's isolated from normal and malignant nuclei. The chemical nature of the DNA-P antigen was confirmed by enzymatic susceptibility and specific absorption spectra.

(5) Monospecific globulins were prepared by absorbing anti-nuclear sera with various normal organ homogenates, blood group cells and different nuclear subfractions. By immunodiffusion, glycine extracted DNA-P gave one line of precipitation with homologous monospecific globulin in an appropriate ionic environment. Furthermore, DNA-P from normal nuclei did not react with monospecific anti-malignant nuclear globulin and, conversely, the DNA-P from malignant nuclei was unreactive with the monospecific anti-normal nuclear globulin. Nuclear subfractions I and II only revealed quantitative differences in antigenic contents in gel diffusion experiments.

(6) Differential reactivity between DNA-P isolated from normal and malignant nuclei was also demonstrated through following the reactions between specific antigen and antibody by ultraviolet spectrophotometry. However, neither native nor denatured DNA isolated from both sources revealed such differential reactivity. Similarly, differences at the nucleotide level could not be detected by hemagglutination tests using synthetic polymucleotides as antigens.
Characterization of antibodies to malignant DNA-P in monospecific serum was accomplished through Sephadex gel filtration, sucrose gradient ultracentrifugation and 2-mercaptoethanol treatment. Antibody activity as assayed by hemagglutination and ultraviolet spectrophotometry was found to be associated with both IgM and IgG globulin fractions.
CHAPTER VI

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