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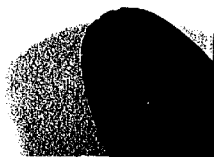
THE REACTION OF CERTAIN BIOLOGICALLY ACTIVE MATERIALS WITH CELLS DERIVED FROM NORMAL AND MALIGNANT TISSUES OF THE RAT

by
Gerard Van Halsema

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THE REACTION OF CERTAIN BIOLOGICALLY ACTIVE MATERIALS WITH CELLS DERIVED FROM NORMAL AND MALIGNANT TISSUES OF THE RAT

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The work reported in this study was undertaken in an attempt to ascertain some cell surface differences between cells derived from normal and malignant tissues of the rat. Our knowledge of the relatively complex nature of the human erythrocyte cell surface is suggestive of the probable complex nature of the surfaces of cells derived from other tissues of mammalian origin. Any antigenic changes which may occur in the tumor cell may or may not be reflected in the make up of the individual cell surface. If they are, it would seem that their detection should be accomplished by serological means. If, however, such differences are not detectable serologically, these and various other cell surface differences might be detected between cells derived from normal and malignant tissues, if means for such detection were available. In this present study, an attempt was made to detect such cell surface differences, employing as a means of detection biologically active substances such as specific antiserums, phytohemagglutinins, and the PR8 strain of influenza virus. Reactions of normal and malignant cells of the rat with these substances were noted.

The minimal amount of tumor material necessary to produce a maximum number of tumor "takes" in rats was also determined.

Three of the five phytoagglutinins used were shown to be capable of agglutinating the rat red blood cell, but only one of the plant extracts was able to actively agglutinate rat white blood cells. Even though tumor cells were not actively agglutinated by any of the phytoagglutinins, it was shown that these cells removed the rat erythrocyte agglutinin from solution, but that they did not remove from solution the chicken erythrocyte agglutinin. The plant extract obtained from the white navy bean (Phaseolus vulgaris Linnaeus) was shown capable of markedly interfering with the viability of the tumor cell in vitro. Adsorption tests showed the tumor inhibiting fraction to be associated with the rat erythrocyte agglutinin. No effect on tumor growth was noted when this extract was given in vivo as a therapeutic measure.

Suspensions of both brain tissue and tumor tissue were shown to be capable of completely adsorbing a standard amount of influenza virus, while rat leukocytes, erythrocytes, and kidney tissue were shown to be incapable of doing so. Active influenza virus was also shown capable of producing a maximum amount of protection against tumor growth, even after partial elution of the virus had occurred. Inactivated influenza virus did not protect against tumor growth, nor did this virus elute from the tumor cell. Given in vivo as a therapeutic measure, the virus was shown to be incapable of producing protective action against tumor growth.

No serological cross reactions were noted in vitro between the rat erythrocyte, leukocyte, and the tumor cell. Protection tests in animals indicated that the antiserums developed against a pellet fraction of tumor tissue were superior to those antiserums developed against tumor homogenates and free tumor cells. However, antiserums developed against both rat erythrocytes and leukocytes were also able to protect against tumor growth. Antiserums developed in and collected from rats which were naturally immune to tumor inoculations were not able to protect against tumor growth. It was also shown that "normal" pre-immunization serums which were not subjected to heat inactivation prior to use were capable of inactivating tumor cells in vitro. This cytotoxic property was shown to be due to a heat labile substance present in these serums.

An aqueous tumor extract was shown to be capable of inhibiting influenza virus hemagglutination in high titer. Tiselius electrophoretic patterns indicated that the extract contained at least four and possibly six components. These components were characterized as to their electrophoretic mobilities and their relative concentrations.

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INTRODUCTION

The serological relationships existing between homologous normal and neoplastic tissues have been investigated by many workers. The underlying basis for their approach has been based on the assumption that apart from the antigens found in normal tissues, there may be an antigen or a constituent which is peculiar to the neoplastic cell, since malignant tissues may be made up in part of altered normal elements. While tumor tissue and homologous normal tissues undoubtedly have a large number of antigenic substances in common, evidence for a specific antigen which is characteristic of the tumor cell, has been difficult to obtain.

The assumption that a change in antigenic structure may occur in the case of the tumor cell is not unfounded. Changes in the antigenic specificity of cells during normal development may occur, as, for example, the change from species to organ specificity in the developing lens (1). Further, there are also recorded instances which indicate the occurrence of changes in the serological specificities of cells in pathological conditions, as evidenced by the appearance of auto-antibodies in the case of liver disease, where antibodies can be detected against the pathologically altered liver, but which are not reactive with normal liver (2).

Our knowledge of the relatively complex nature of the human erythrocyte cell surface is suggestive of the probable complex nature of the surfaces of cells derived from other tissues of mammalian origin. Any antigenic changes which may occur in the tumor cell, however, may or may not be reflected in the make up

of the individual cell surface. If they are, their detection should be accomplished by serological means. If, however, such differences are not detectable serologically, these and various other cell surface differences might be detected between cells derived from normal and malignant tissues, if means for such detection were available. In this present study, an attempt was made to detect such cell surface differences, employing as a means of detection such biologically active substances as specific antiserums, phytohemagglutinins, and the PR8 strain of influenza virus.

HISTORICAL

Despite the repeated attempts that have been made by many investigators to obtain an antibody specifically reactive against tumor tissue, much of the evidence recorded in the voluminous literature supports the current view that, apart from those tumors of known viral etiology, no conclusive evidence exists which indicates any qualitative differences in antigenic composition between homologous normal and malignant cells. Heading a U. S. Public Health Service Commission in 1938, S. Bayne-Jones reported that "attempts to establish fundamental differences between normal and malignant cells of the same tissue type have failed to bring out any striking variations in chemical make up, enzyme content, metabolism or structure" (3).

The picture remains essentially unchanged today. On the other hand, there are also to be found various instances in which claims for the detection of qualitative differences between normal and neoplastic tissues are amply documented with experimental proof. These, however, are comparatively few in number, since most of the work done on the serological relationships between the two types of tissue points to the antigenic similarities existing between the tumor and the normal cell.

Many workers have shown that antigenic similarities do exist between normal and malignant tissues. Itami (4, 5) conducted an investigation to determine the immunizing power of ectodermal, mesodermal, and endodermal derivatives against transplantable tumors in mice. It was found that representative tissues from each of these

groups were capable of inducing resistance to tumor growth. Animals inoculated with skin, blood, liver, and many other tissue derivatives all exhibited a certain degree of immunity to subsequent tumor inoculations. Barrett and Hansen (6, 7) showed that resistance to implantation of a mouse sarcoma in an inbred line could be induced by the inoculation of defibrinated blood or fractions of blood from unrelated mice, with the effective antigen being found in the red cells and not in the serum. It is interesting to note that in contrast to the findings of Itami, Barrett found the immunizing power against tumor growth to be associated with the red blood cells. Itami, however, observed that the leukocytes and lymphocytes were responsible for inducing tumor immunity to a marked degree, and that the erythrocytes were incapable of producing this effect.

Bashford et al (8) showed that mice in which a growing carcinoma had spontaneously regressed may be completely protected against subsequent inoculations of the same type of tumor, and that this protection could also be produced by the inoculation of homologous normal tissues, including blood. It was again shown that this immunity was conferred by the blood cells and not the serum. The blood of related animals, i.e., rat, rabbit, guinea pig, elicited no such protection.

Haaland (9) demonstrated that the inoculation of living, intact tumor cells or normal tissue cells into susceptible animals was capable of producing an immunity to subsequent tumor inoculation, whereas the inoculation of mechanically ruptured tumor cells or normal cells was not able to produce this immune reaction. Barrett

and Hansen (6, 7) observed also that whereas intact red cells were able to produce an increased resistance to sarcoma growth, laked cells would not bring about this reaction, and therefore it was postulated that tumor immunity induced by the red cell antigen is based on the architectural integrity of the cell surface or some major part of it.

Sturm (10) observed resistance to transplantable lymphatic leukemia in rats by the prior injection of embryonic tissue into these same animals. Rhoads and Miller (11) have also been able to induce resistance to transplantable leukemia in mice by the injection of normal homologous tissues, such as normal spleen and lymph node suspensions.

MacDowell and others (12, 13) were also able to produce immunity to leukemia in susceptible mice not only with sublethal doses of leukemic cells, but also with normal tissues from a foreign strain. The only difference between these two types of immunity was that the immunity of the mice which were immunized to leukemic cells was transferable to normal mice by the injection of splenic or hepatic tissues from the immune animals, whereas the immunity developed by foreign normal tissue was not transferable.

Still other workers have presented evidence for the existence of antigenic similarities between malignant and normal tissues. Phelps in 1937 (14) developed antiserums to both Jensen rat sarcoma and to normal spleen tissue, and claims to have observed in tissue culture that the antitumor serum killed the normal as well as the malignant cells. Moreover, it was also asserted that the antispleen

serum killed malignant as well as spleen cells, and in addition, the antispleen serum exhibited a more potent autolytic effect on tumor cells than did the antitumor serum. He concluded from this work that the tumor tissue did not contain a tumor specific antigen at all, and that the non-specific action of the antitumor serum on normal cells could be attributed to the fact that the malignant tissue contained antigens which were similar to those present in the spleen tissue, and that these gave rise to antibodies identical with those present in the antispleen serum.

Gorer (15) was successful in producing an antiserum to leukemic cells in the resistant C57 black strain of mice, as shown by the fact that an antibody was obtained which was capable of producing a definite retardative effect on growth of the leukemic cells transplanted into mice. The leukocytes as well as the red blood cells of the susceptible albino mouse were capable of adsorbing this antibody, indicating that similar antigenic components exist on the erythrocytes, white blood cells, and the leukemic cells of the albino mouse.

There are also many well documented in vitro serological studies on the relationships between tumor and normal tissues. Steinberg and Martin (16) demonstrated that mature and immature lymphocytes and polymorphonuclear leukocytes possess individual specific antigenic components as demonstrated by the specific agglutination of each cell type. The presence of specific cell antigens for lymphocytes and granulocytes in their mature and immature forms, therefore, connotes a different chemical composition for the surface of each

type of cell. There was no differentiation of cell specificity for leukocytes obtained from normal and leukemic people, suggesting that leukemic leukocytes are similar in chemical structure to normal leukocytes.

The studies of Dulaney and Arnesen and others (17, 18, 19) showed that serological relationships exist between the mitochondria and submicroscopic particle fractions segregated from the spleen, liver, and kidneys of normal and leukemic mice, indicating that these fractions of the tissues studied contain cross reactive antigens.

Dmochowski (2) was not able to detect any serological reaction in animals against their own malignant cells, indicating that primary spontaneous tumors do not possess specific antigenic properties which are different from those belonging to normal tissues. By the injection of saline extracts of these tumors into rabbits, antiserums were produced which fixed complement in the presence of homologous tumor extracts. These serums were also capable of fixing complement, though in a lower titer, with extracts of normal rat testicle, stomach, intestine, lung, and rat embryo. Alcoholic extracts of these same tissues also gave results which indicated that no antigenic differences existed between tumor and normal rat tissues. These same antitumor serums, adsorbed with tumor tissue, no longer cross reacted with extracts of lung, stomach, and embryonic tissue. Immune serum against normal rat muscle contained antibodies which fixed complement with rat tumor extracts even more strongly than with the homologous antigen. Dmochowski was thus able to detect only quantitative differences between extracts of tumor and normal

tissues, the cross reactions between the two being explained by the presence of common normal antigens in these tissues.

d'Alessandro (20) also found only quantitative differences in antigenic composition between tumor tissue and normal tissues. He found that immune serum prepared against Jensen sarcoma extracts reacted with extracts of homologous normal organs, such as stomach and intestines.

Earlier German workers attempted to show serologically detectable differences between human cancer tissue and normal human tissue. Hirszfeld et al (21, 22) claim to have developed cancer specific antiserums which fixed complement with extracts of gastric tumors and other tumors of the digestive tract, and which rarely gave cross reactions with normal organs. In some cases, however, the cancer specific antigen was also obtained from normal organs, where no cancer could be detected macroscopically. Hirszfeld in such instances did not believe that similar antigenic components existed between the cancerous and the normal tissue, but postulated a "biochemical infiltration" or "biochemical metastasis" from the cancer into the neighboring tissue. He explained that in cancer, a disturbance in the lipoid metabolism occurs in the affected tissue, with the resulting formation of a differentiated lipoid which is characteristic of the tumor antigen.

Hirszfeld and his coworkers (23) also carried out a series of experiments in which they attempted to observe reactions between group specific immune serums and alcoholic extracts of human cancerous and normal tissues. The carcinomas studied were found

to nearly always contain group A substance, whereas the sarcomas did not show the presence of this isoagglutinogen. It was also found that the liver of persons belonging to blood group A frequently possessed isoagglutinogens when there was a cancer growing in the vicinity of the liver even though the presence of the group substance in the liver was not determined by a visible metastasis of the cancer into the liver. It was again postulated that the presence of A substance in such cases rested upon an imbibition of dissolved substances derived from the cancer. Extracts of tumors from persons belonging to blood groups O and B reacted frequently with anti A serum. Hirszfeld calls these "serological malformations".

These same workers (24) obtained evidence of the presence of similar antigens in human cancerous tissue, leukocytes, and pus. Hirszfeld considered that the antigenic similarities between tumor cells and leukocytes might depend on similarities in their life spans; leukocytes are among the shortest lived of body cells, and tumor cells supposedly grow and die more rapidly than normal cells. The indication was that the antigenic properties of cancer tissue, which may distinguish it from normal tissues, may be due to rapidly dying tissues, and the question was raised as to whether necrotic tissue as such has a peculiar constitutional specificity or whether these properties are due to secondary leukocytic infiltration.

In another series of experiments, Hirszfeld and his colleagues (25) found that antiserum against rat embryo extract was capable of fixing complement with alcoholic extracts of rat placenta and also alcoholic extracts of human tumors. They found that immune serum

against normal rat organs reacted more strongly with human tumor extracts than with extracts of normal rat organs.

Witebsky and Morelli (26) also studied the question as to whether human malignant cells contain a modified organ antigen. With the complement fixation reaction they found that there were marked cross reactions between alcoholic extracts of spleen and sarcoma with serum from rabbits immunized with sarcoma cell suspensions. The sarcoma antibody was readily adsorbed by extracts of normal organs. Cross reactions were also noted with this serum between sarcoma and carcinoma tissue extracts. Antiserum prepared against alcoholic extracts of heterologous tumor tissue from the rat and hen did not cross react. Witebsky and Poplau (27) also were not able to develop a specific antiserum against malignant tissue, since the serum which they obtained showed an ability to react also with extracts of normal organs, notably the intestine.

Maculla (28, 29, 30) made an extensive investigation into the immunological relationships existing between various mouse tissue components derived from organs of adult and embryonic mice, including a number of transplantable tumors. Using the complement fixation technique, it was demonstrated that the eight tumors which she used possessed intracellular antigenic components in common with adult mouse spleen and lung. Some tumors also appeared to possess components which were distinct from those of normal tissues. The nucleoprotein fraction obtained from mouse tumors was immunologically distinct from the nucleoprotein fraction of normal mouse organs. However, some tumors were found to possess closely related components

and in addition, six of the tumors studied appeared to possess components common also to embryonic liver.

Gorer (31, 32) reported the existence of antigenic differences in the red blood cells of different inbred strains of mice. These antigens were found not only in the erythrocytes but also in the homologous tumor tissue as well. The transplantability of the tumor in question was dependent not only on the presence of these antigens in the tumor tissue but also in the normal tissues of the host. He observed that Strong's A strain of albino mice, a strain highly susceptible to cancer, possesses two isoantigens that are shared by the animal's erythrocytes and a number of its fixed tissues, including all the neoplastic tissues studied in this host. Successful transfer of the sarcoma studied was accomplished in any other member of the inbred line, but the tumor could not be transferred to unrelated strains of mice.

Lumsden's work (33) also showed that the successful transplantation of the Jensen rat sarcoma was dependent on the presence of an antigen in the sarcoma which was identical with an antigen present in the recipient animal's erythrocytes; if the erythrocytes did not contain this isoantigen, the inoculated animals did not develop progressive tumor growth. Further, Eisen and Woglom (34) and Barrett (35) have also demonstrated that acquired resistance to transplantable tumors depends upon genetic differences between the animal inoculated and the one that originally produced the neoplasm, which was in turn again associated with the presence of a common antigen in the tumor tissue and in the animal's erythrocytes.

Apart from those instances which record the production of antibodies against tumors of viral etiology, there are also to be found several recorded instances which deal with the production of so-called specific antitumor serums. Evidence of this kind gives an indication of the possible qualitative differences which may exist between normal and neoplastic tissues.

Malmgren (36), using the complement fixation reaction, made a serological study of the microsome fractions from normal and neoplastic mouse tissues. Quantitative differences were noted between these fractions obtained from normal liver and hepatoma tissue. However, a comparison of the microsome fractions from mammary tumor and hepatoma tissue revealed qualitative differences that were interpreted as indications of organ specificity. The differences were not attributable to the mammary milk agent since this particle was not detectable in the microsome fraction.

Bunting (37) also obtained results which indicated an antigenic dissimilarity to exist between malignant mouse tissue and normal mouse organs. He observed that antitumor serum did not react with most antigens of various normal mouse organs: liver, kidney, brain or mouse serum. The specific antigenic substance was also shown to be lipoidal, being soluble in ether and alcohol.

Nettleship (38) has observed the regression of the Murphy lymphosarcoma by the injection of heterologous antibodies. By the use of rabbit antilymphosarcoma serum, it was possible to produce both retardation in rate of tumor growth and a significant reduction

in mortality in rats bearing the tumor. It was found necessary to utilize serum of high titer and in considerable quantity to effect these results. Similarly, Nungester and Fisher (39) have observed protection against the growth of a lymphoblastoma in an inbred strain of mice by the injection of heterologous antibodies. Immune serum developed in rabbits against a sedimentable tumor pellet fraction afforded this protection, whereas, a like fraction obtained from normal tissues of non-inbred mice failed to give protection.

Werder et al (40) undertook a study to demonstrate differences in the antigenic components of mouse leukemic cells and of normal mouse tissue. They were able to show that when a lethal dose of leukemic cells for mice was mixed with a homologous antibody, the mice were protected in 80-100% of the cases. However, this same protection was afforded with antispleen tissue serum, with the difference that the protective substance found in the antileukemic cell serum was heat stable, whereas the factor in the antispleen serum was heat labile.

The serological relationships existing between tumor tissue and normal tissues are thus apparent. The evidence that definite antigenic similarities do exist between these two types of tissue in contrast to the evidence that definite qualitative differences might also exist, is conflicting and it is thus difficult to draw any definite and final conclusions as to the antigenic specificity of neoplastic tissues.

In 1950, Nungester et al (41) employed other than purely serological means to compare the cell surfaces of the guinea pig

erythrocyte and leukocyte. By the use of agglutination techniques, reactions of both types of cells were noted with such agglutinating substances as specific antiserum, PR8 strain of influenza virus, pneumococcus polysaccharide, and a phtyoagglutinin. Definite similarities and differences were shown to exist on the surfaces of these two types of cells as determined by their respective reactions with these substances.

The use of phytoagglutinins as blood grouping reagents has been suggested by Boyd (42, 43). In considering Landsteiner's work with plant extracts (44, 45) he also noted that dilute extracts of various plants exhibited different degrees of agglutination with erythrocytes of different species. In particular a number of plants yielded extracts which were specific and highly active for blood group A. It was therefore of interest in the present study to note any differences of reaction between cells derived from normal and malignant tissues of the rat with certain of these phytoagglutinins. Any differences of reaction would suggest that cell surface differences do exist between these two types of cells.

In addition, the production of untoward effects on tumors and tumor growth by various plant extracts has been the concern of many workers. A compilation of negative data obtained by these investigators engaged in screening various compounds for their effect on neoplastic growth was made by C. Chester Stock (46) of the American Association for Cancer Research. Included in this publication are the results obtained in determining the effect of many plant extracts on various types of tumors. Other reports in

the literature include the work of Taylor et al (47), in which the inhibition of the growth of egg cultivated tumor tissue was noted by extracts of Cooperia pedunculata herb. Also, Guyer and Claus (48) reported the destructive effects of colchicine on rat tumors, and Lewisohn and coworkers (49) found that yeast extracts adversely affected spontaneous breast adenocarcinomas in mice.

As mentioned previous, Nungester et al (41) employed the PR8 strain of influenza virus as a means of detecting cell surface differences between the guinea pig leukocyte and erythrocyte. Hirst (50, 51) has shown in his studies on influenza virus hemagglutination that an adsorption of the virus to the cells is necessary to produce this clumping, and that definite "receptor areas" on the cell surface are involved in the adsorption. It was also shown that elution of the virus was associated with the destruction or inactivation of these receptors. It is thus apparent that informative comparisons of the respective abilities of normal and neoplastic cells to adsorb this virus would point to the existence of either cell surface differences or similarities.

Much work has been done in an attempt to characterize the virus particle itself and also the red cell receptor areas. Hoyle (52) states that the influenza virus particle can be disintegrated into two fractions, one of which is the soluble immunizing antigen and the other the red cell agglutinin. The agglutinin is an enzyme with a protein part carrying the enzymic activity and a combining group with affinity for red cells. The character of the virus enzyme is not yet well understood, but it is thought to be closely related to

soluble enzymes of similar action found in culture filtrates of several bacterial species, notably those of Cl. welchii (53) and Vibrio cholerae (54, 55). The substrate found on the red cell surface is probably made up of macromolecular carbohydrate amino acid compounds. The action of the enzyme is not to break up the macromolecules but to split off functional groups, of which there are several in each macromolecule (56). According to Gottschalk (57, 58, 59), these functional groups are probably N substituted isohexosamine units which are attached to the molecule through a hexose unit. He also found that purified mucoprotein was capable of inhibiting influenza virus hemagglutination. Many substances capable of inhibiting viral hemagglutination are present in a wide variety of physiological secretions and tissue extracts containing mucoid material (60). Much of the evidence points to the mucoprotein components of such materials as being the active agents in inhibiting hemagglutination by the PR8 strain of influenza virus (61, 62).

deBurgh and his colleagues (63) have attempted to isolate and characterize the receptor or substrate on the red cell surface by chemical extraction procedures and have concluded on the basis of their study that this receptor area or part of it is polysaccharide in nature.

Moore and her collaborators have done a considerable amount of work in studying the effect of viruses on tumors with a view to finding a viral agent which has a specific destructive action on tumors in general. In one of Moore's original publications (64)

she reported that both viruses influenza A and Herpes simplex could be detected for a limited period of time when inoculated directly into sarcoma 180, but that their presence in the tumors had no effect on tumor growth. With another of the hemagglutinating viruses, Moore (65, 66) found that when suspensions of sarcoma 180 cells and Ehrlich carcinoma cells were mixed with Newcastle Disease virus, there was a fall in hemagglutinating titer of the virus, indicating an adsorption of the virus to the tumor cells. Associated with this decrease in hemagglutinating titer was an inhibition of tumor growth. When the mixtures were allowed to stand at room temperature for several hours, there was an increase in the hemagglutinating titer of the virus with an accompanying increase in tumor growth. Kausche et al (67), using the PR8 strain of influenza virus, noted a reduction in the growth of Ehrlich ascites mouse tumors when this virus was injected into the mice prior to tumor inoculation.

Earlier investigators put more emphasis on the growth of the virus in the tumor rather than the effect of the virus on tumor growth. It was often noted that upon transplantation of the virus-infected tumors into new animals, there was a concomitant decrease in the incidence of tumor growth (68 - 73). Only recently have various workers taken up an intensive study of the effect of viruses on tumors. It has been found that for the adverse tumor effect to be produced, active virus must be present, and this effect therefore will not take place in animals immune to the virus or with inactivated virus. In addition, each virus appears to differ considerably in its tumor destroying ability, indicating that oncolysis by viruses is a specific affair (74).

Ackerman and Kurtz (75) in adapting the WS strain of influenza A virus to a mouse ascitic tumor found that associated with multiplication of the virus there was a loss of viability in the host cell. Southam et al (76) also demonstrated a temporary inhibition of leukemic cytolysis in the late stage of mouse leukemia with the West Nile and Ilheus viruses. Buckley et al (77) have also shown that the Webster No. 3 strain of St. Louis Encephalitis virus multiplies in and has an adverse effect upon the growth of the Crocker mouse sarcoma 180. Ginder and Friedewald (78) have demonstrated the disappearance of the rabbit fibroma by the intramuscular inoculation of Semliki forest virus, and have also indicated the significant reduction in myxoma rabbit fatalities by the intramuscular injection of the same virus.

Koprowski and Norton (79) made a study of the infection of transplantable mouse tumors with certain neurotropic viruses, such as Louping Ill, West Nile, Ilheus, Russian Spring Summer, St. Louis encephalitis, etc., on the viability of sarcoma 180 tumor cells and found that different viruses had varying effects on tumor growth. Their results indicate that it is unwise to generalize on the behavior of viruses in relation to all transplantable tumors, and that one should confine an analysis of such data to the effect of a particular virus on one particular type of tumor without drawing general conclusions.

Love and his colleagues (80, 81) also determined that of the various viruses with which they worked, West Nile and Bunyamwera viruses were the only ones that had a pronounced inhibitory effect

on ascitic tumors in mice. Moore (82) has shown five transplantable mouse tumors of different histological types to be completely destroyed during the course of infection of the host animal with the virus of Russian Far East encephalitis. She has also shown that this same virus is capable of destroying sarcoma 180 in mice, and that destruction occurs irrespective of the age of the tumor and the route of inoculation (83 - 86). Southam and Moore (87) used Egypt 101 virus clinically in 34 patients with advanced neoplastic disease. In no instance was it observed that this virus exerted a curative effect, but in many instances the virus was found to be localized in the tumor tissue.

EXPERIMENTAL

I. Materials and Methods

A. Source, Growth, and Preparation of Tumor Material

The Flexner - Jobling rat carcinoma which was used as a source of tumor material in these experiments was obtained from Dr. G. A. LePage of the McArdle Memorial Laboratory at the University of Wisconsin.

The tumor was originally discovered in 1906 by Drs. Flexner and Jobling at autopsy of an adult, male stock rat. It was originally regarded as a sarcoma, but in the course of transplantation it was observed to pass through several changes in structure, and it finally became established as an adenocarcinoma (88). The pathology of the tumor used currently in this laboratory has been identified as a "poorly differentiated carcinoma, possibly of the squamous type."* The tumor was routinely maintained in albino stock rats by the subcutaneous inoculation of 0.5 ml of a 5% tumor cell suspension, which usually gave rise to visible tumors within 10 - 15 days. The tumor growth attained an average size of approximately 10 - 15 mm. in diameter within this time. The tumor could also be grown intraperitoneally, in which case the tumor growth was accompanied by the marked production of bloody ascites, with visible metastases on the peritoneum. Generally, death in the case of intraperitoneal tumor growth occurred much more rapidly than in the case of subcutaneous tumor implantations.

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* We are indebted to Dr. D. L. Hinerman for the pathological diagnosis of this tumor.

All rats used were between 150 - 200 grams in weight and were of the Sprague-Dawley strain. The tumor was observed to grow equally well in members of both sexes. All rats were fed a complete dog ration, "Friskies".

Tumor material used in experiments was usually obtained from subcutaneous tumors which were excised between 12 - 15 days after inoculation. Tumor cell suspensions were made under aseptic conditions by removing the connective tissue from the mass of tumor, mincing the tumor with scissors, and grinding the tissue in a Potter-Elvejhem type homogenizer in an isotonic serum-substitute solution described by Krebs (89). The homogenate was then filtered through 20 XX mesh nylon, the filtrate centrifuged, and the sediment washed twice in Krebs' solution. This was followed by resuspension of the cells to the desired concentration on the basis of volume of packed cells. Microscopically, the suspension was seen to contain many, whole single cells, with an occasional group of 2-3 cells also present. In addition, ruptured cells and connective tissue elements were probably observed as unidentifiable debris. Intact cells were assumed to be tumor cells. A 5% suspension of this tumor material contained 10,000 - 12,000 of these whole cells per cu. mm.

B. Erythrocytes and Leukocytes

Rat erythrocytes were obtained in citrate solution from albino stock rats by cardiac puncture. The cells were washed three times in saline solution and were resuspended to the desired concentration on the basis of volume of packed cells. Chicken

erythrocytes were also collected in citrate solution from adult roosters by cardiac puncture and treated in a similar manner.

Rat leukocytes were obtained by harvesting the peritoneal exudate from adult albino rats, each of which had been inoculated 10 - 12 hours previously with approximately 100 ml of Locke's solution containing 0.1% glucose. The cells thus obtained were washed three times in Locke's solution and the desired concentration of white cells was made on the basis of volume of packed cells. Microscopically, most of these cells were observed as polymorphonuclear leukocytes.

C. Normal Brain Tissue

The craniums of normal stock rats were opened aseptically and the brains removed. The brain tissue was washed in sterile saline to free it from blood, and then minced and ground to a 20% suspension by weight. The brain tissue was then washed three times in sterile saline solution, and finally it was resuspended to a 2.5% concentration on the basis of volume of packed tissue.

D. Normal Kidney Tissue

With aseptic technique the kidneys of normal stock rats were exposed and perfused in situ with saline solution through the renal artery. After removal, the kidneys were minced and ground to a 10% suspension in saline solution. The tissue was placed in distilled water for 30 seconds to hemolyze the remaining blood cells, and was then washed three times in saline solution. The tissue was resuspended to give a 5% suspension on the basis of volume of packed tissue.

E. Source and Preparation of Phytoagglutinins

Four different plant agglutinins were obtained from Dr. W. C. Boyd of the Boston University School of Medicine, Boston, Massachusetts. These phytoagglutinins were lyophilized preparations of saline extracts obtained from various bean plants. They were reconstituted in saline solution to a concentration of 10 mgm per ml. These were designated as stock solutions and were kept at 5°C. Material for experimental work was taken from these stock solutions and was diluted 1:10 in saline solution before use in agglutination tests. In addition, a fifth phytoagglutinin was made from white navy beans after the method of Li and Osgood (90). The only modification of their procedure was that the extract was adjusted to a pH of 6.0 with 20% NaOH. The action of these phytoagglutinins against human red cells is as follows:

Phytoagglutinin No.	Source	Type of Human Erythrocyte Agglutinated
1.	Phaseolus limensis Macfadyen var. "Westside Lima"	A
2.	Phaseolus vulgaris Linnaeus var. "Golden Cluster Wax"	A, B, O
3.	Phaseolus limensis Macfadyen var. "Finast Lima"	A
4.	Phaseolus vulgaris Linnaeus var. "Rustproof Golden Wax"	A, B, O
5.	Phaseolus vulgaris Linnaeus var. "White Navy Bean"	A, B, O

F. Antiserums

1. Rat Erythrocyte Antiserum

Antiserum to rat erythrocytes was prepared by the intraperitoneal inoculation of 1.0 ml of a 20% suspension of red blood cells into rabbits thrice weekly for two weeks, followed by the intravenous inoculation of 0.5 ml of a 1% suspension of red blood cells daily for one week. These animals were then bled by cardiac puncture one week after the last injection of erythrocytes.

2. Rat Leukocyte Antiserum

Antiserum to rat leukocytes was prepared by the intraperitoneal inoculation of 1.0 ml of a 5% suspension of leukocytes into rabbits thrice weekly for two weeks, followed by the intravenous inoculation of 0.5 ml of a 1% suspension of leukocytes daily for one week. The rabbits were bled by cardiac puncture one week after the last injection of antigen and the serum obtained. Hyperimmune serum was obtained in these same animals after three months by the intravenous inoculation of 1.0 ml of a 5% suspension of white blood cells twice weekly for three weeks. Serum was again obtained after the final injection of antigen in the same manner as outlined above.

3. Tumor Antiserum

Three types of antiserums to tumor tissue were prepared in rabbits by means of three different immunizing preparations.

a. Free tumor cells were obtained from ascitic fluid developing with intraperitoneal tumors. The ascitic fluid was

centrifuged and the cells treated with a 1% saponin solution to lyse the red blood cells, and the sediment obtained was washed three times with saline solution. Microscopic examination of the sediment revealed various forms of white blood cells along with other cells sometimes grouped in clumps of three or four cells, which were tentatively identified as tumor cells. Inoculum prepared from this sediment gave rise to subcutaneous tumors more rapidly and with more visible metastases than inoculum prepared from subcutaneous tumors. A 20% suspension of these cells was mixed in a 1:1 ratio with Salk adjuvant (91) and this antigen-adjuvant preparation was injected intramuscularly into the thighs of rabbits, 1.5 ml per site, 4 sites per animal. Six weeks after inoculation of this mixture the rabbits were bled by cardiac puncture and the serum collected.

b. Tumor homogenate was prepared from 12 - 15 day old subcutaneous tumors which were homogenized as described in (a) above and made up to a 20% suspension. This suspension was then prepared as an antigen with adjuvant as described in (a) above, and was given in the same dosage and by the same route of inoculation. Six weeks after inoculation of this mixture the rabbits were bled by cardiac puncture and the serum obtained. Hyper-immune serums were also obtained in the case of these animals by immunizing with tumor homogenate in the same manner as before, after the first and second bleedings.

Also, ten rats which were found to be naturally immune to tumor inoculation were used for immunization purposes. These rats

were given 4.0 ml of a 5% suspension of tumor cells intraperitoneally twice a week for three weeks. One week after the last immunizing injection the animals were bled by cardiac puncture and the serum collected.

c. Tumor pellet fraction was prepared from a known weight of subcutaneous tumor which was minced, homogenized and resuspended to a 20% concentration. This suspension was then centrifuged at 4000 rpm for 40 minutes, and the supernatant fluid thus obtained was then recentrifuged at 13,000 rpm for 90 minutes. Both centrifugations were made in a Serval angle head centrifuge at 5°C. The pellet obtained after the second sedimentation was resuspended in a total volume of saline solution which was twice the amount of the original starting weight of the tumor material. This was again homogenized, filtered through nylon, and the filtrate was then used as the antigen. Two rabbits were immunized against this fraction by the injection of 5.0 ml intraperitoneally twice weekly for three weeks, followed by a final injection of 10.0 ml by the same route. The animals were then bled by cardiac puncture one week after the last injection and the serum collected.

4. PR8 Influenza Virus Antiserum

Each of two adult roosters was given 5.0 ml of a 1:1 mixture of infected allantoic fluid - Salk adjuvant intramuscularly, plus 5.0 ml of infected allantoic fluid intraperitoneally. Three weeks after the inoculation of the virus, these roosters were bled by cardiac puncture and the serum obtained.

All serums used were inactivated at 56°C for 30 minutes unless indicated otherwise.

G. Source and Cultivation of Influenza Virus

The PR8 strain of influenza virus A was obtained from frozen stock of infected allantoic fluid. This frozen material was thawed, and a 1:100 dilution of this virus suspension was made in saline solution. 0.25 ml of the diluted virus was inoculated into the allantoic cavity of a 9-10 day old embryonated chicken egg. The allantoic fluid was harvested and pooled after 40-48 hours incubation at 37°C. This pooled allantoic fluid usually exhibited a hemagglutinating titer of 1:4096 with 0.25% chicken red blood cells, and in cases in which a higher titer was obtained, an appropriate dilution of the infected allantoic fluid was made prior to its use to give a titer of 1:4096. Normal allantoic fluid was collected from uninoculated embryonated eggs of the same age and also pooled.

H. Hemagglutination Tests with Influenza Virus

All hemagglutination tests and hemagglutination inhibition tests were conducted according to the method described by Salk (92). The hemagglutination inhibition tests were carried out by adding two hemagglutinating units of virus to each serial dilution of inhibitor and chicken erythrocytes. Tests were read by noting the settled red blood cell pattern deposited on the bottom of serological tubes of uniform curvature.

I. Adsorption-Elution Experiments with Influenza Virus

To three volumes of a 5% suspension of tumor cells was added one volume of a standard amount of influenza virus in allantoic fluid. At fixed intervals of time aliquots of this mixture were removed, centrifuged, and the supernatant was assayed for the presence of virus with chicken red blood cells. Doubling serial dilutions of the supernatant were made in saline, and to each dilution in the series was added an equal volume of 0.25% chicken erythrocytes to give a final series dilution of 1:8192. Results of hemagglutination were read at the end of two hours at room temperature.

J. Protection Tests in Rats

1. Protection tests with phytoagglutinins were carried out by the in vitro mixing of 3.0 ml of a 5% suspension of tumor cells and 3.0 ml of the stock solution of plant extract. This mixture was placed on a rotary shaker at room temperature for two hours, subsequent to which each of 5 rats was inoculated subcutaneously with 1.0 ml of each mixture, 2 sites per animal, 0.5 ml per site. Tumor growth was recorded every fifth day through twenty days.

2. Protection tests with influenza virus were carried out simultaneously with an adsorption-elution experiment. Three volumes of a 5% suspension of tumor cells was mixed with one volume of a standard amount of virus in allantoic fluid, resulting in a final virus dilution of 1:4. At fixed intervals of time, aliquots of this mixture were removed and each of 5 rats was

inoculated subcutaneously with 1.0 ml of each aliquot, 2 sites per animal, 0.5 ml per site. Tumor growth was recorded every fifth day for twenty days.

3. Protection tests with antiserums were carried out by the in vitro mixing of 3.0 ml of a 5% suspension of tumor cells and 3.0 ml of an undiluted specified serum. This mixture was placed on a rotary shaker at room temperature for three hours, subsequent to which each of 5 rats was inoculated with 1.0 ml of each mixture, 2 sites per animal, 0.5 ml per site. Tumor growth was recorded every fifth day for twenty days. All serums used were heat inactivated at 56°C for 30 minutes unless otherwise indicated.

K. Agglutination Reactions with Antiserums

All serums used in agglutination reactions were serially diluted in saline solution in doubling increments, and to each dilution was added an equal volume of a specified concentration of either rat leukocytes or erythrocytes. The final serum dilution in the last tube of each series was 1:2048. Tests were placed in a 37°C water bath for two hours, subsequent to which the tests were read for agglutination. Reactions were also read after standing overnight at 5°C.

L. Absorption of Serums

Specified serums to be used in agglutination reactions were diluted 1:1 with saline. For absorption of these serums with tumor material, 6.0 ml of a 1:2 dilution of a given serum was mixed with 0.25 ml packed tumor material. Mixtures were placed

at 37°C with intermittent shaking for two hours, and were then kept at 5°C overnight. Agglutination tests were done on these serums with rat erythrocytes and leukocytes, after which these same serums were absorbed a second time in a similar manner with tumor material. Agglutination tests were then repeated on this twice-absorbed serum. For the absorption of serums with rat leukocytes or erythrocytes, the same volume of similarly diluted serum was mixed with 0.5 ml of either packed red blood cells or white blood cells. These were also placed at 37°C for two hours and at 5°C overnight, as described above.

M. Tumor Extract

To 50 grams of freshly excised, minced subcutaneous tumor was added 50 ml of chilled distilled water. This was placed in a Waring blendor for five minutes, and 50 ml of rinse water was added to the mixture. This homogenate was placed on a rotary shaker at 5°C for four hours. The mixture was filtered in the cold, and the filtrate thus obtained was again filtered for sterilization through an ultrafine glass filter. Such material was stored at 5°C, and was used in anti-hemagglutination tests.

N. Electrophoretic Analysis

Analysis of the tumor extract was made in the Tiselius electrophoresis apparatus employing the Schlieren scanning procedure.* Veronal buffer at pH 8.5 was used, which is 0.1 M with

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* The technical assistance in the operation of this apparatus by Mr. M. F. Barile is much appreciated.

respect to the sodium salt and 0.02 M with respect to the diethyl barbituric acid. Samples were dialyzed against this buffer a minimum of 24 hours before analysis. Analysis of this tumor extract was also made electrophoretically on a paper strip in Veronal buffer at a pH of 8.5, at 10 mamp., with an approximate potential gradient of 4.25 V/cm. Protein components were observed after development of the paper with a saturated solution of mercuric chloride in 95% alcohol, to which had been added 0.1 gm % of bromphenol blue as described by Durrum (93).

II. Results

A. Determination of Minimal Dose of Flexner-Jobling Carcinoma for Rats.

Five separate experiments were conducted to determine the minimal amount of tumor material necessary to produce a maximum number of progressively growing tumors in rats. The tumor cell suspensions were prepared as previously described, and all inoculations were made subcutaneously. Results are given in Table I.

It is to be noted from the results that an inoculum of 0.25 ml of a 5% suspension of tumor material is capable of producing tumor growth in 80 - 100% of the sites inoculated.

B. The Reaction of Certain Phytohemagglutinins with Rat Erythrocytes, Leukocytes, and Tumor Cells.

The agglutinating titers of the phytoagglutinins for the cells under consideration were determined by serially diluting in saline solution an aliquot of a 1:10 dilution of the stock solution of each plant extract, and adding to each dilution of the series an equal volume of 1% cells. The agglutination in each case was read after standing at room temperature for two hours. Chicken red blood cells were also tested for their agglutinability with these phytoagglutinins. The resuls of these tests are given in Table II.

It is indicated in this table that phytoagglutinin numbers 2, 4, and 5 actively agglutinate rat erythrocytes, while only number 5 agglutinates rat white blood cells. No agglutination was observed in the case of tumor cells.

TABLE I

Results of Tumor Growth Following the Subcutaneous Inoculation of Graded Doses of Tumor Material

Expt. No.	Group	No. Rats 2 Sites Each	% Suspension	Amount/Site	Tumor Growth 20 Days
1	1	5	5%	1.0 ml	8/10*
	2	5	5%	0.50 ml	10/10
	3	5	5%	0.20 ml	8/10
	4	5	5%	0.10 ml	7/10
2	1	5	5%	0.50 ml	10/10
	2	5	5%	0.20 ml	8/10
	3	5	5%	0.10 ml	4/10
	4	5	5%	0.05 ml	7/10
	5	5	5%	0.02 ml	4/10
	6	5	1%	0.05 ml	2/10
3	1	5	5%	1.0 ml	10/10
	2	5	5%	0.75 ml	10/10
	3	5	5%	0.50 ml	10/10
	4	5	5%	0.25 ml	10/10
	5	5	5%	0.10 ml	7/10
	6	5	5%	0.01 ml	3/10
4	1	5	5%	0.75 ml	10/10
	2	5	5%	0.50 ml	10/10
	3	5	5%	0.25 ml	8/10
	4	5	5%	0.10 ml	4/10
	5	5	1%	0.25 ml	4/10
	6	5	1%	0.10 ml	2/10
5	1	5	5%	0.50 ml	9/10
	2	5	5%	0.25 ml	9/10
	3	5	5%	0.10 ml	4/10
	4	5	1%	1.0 ml	7/10
	5	5	1%	0.50 ml	7/10
	6	5	1%	0.10 ml	0/10

* $\frac{\text{No. of tumor takes}}{\text{No. of sites inoculated}}$

TABLE II

Agglutination Titers Obtained with Phytoagglutinins

Phyto. No.	Rat RBC	Rat WBC	Chicken RBC	Tumor Cells
1	0	0	0	0
2	1:40	0	1:640	0
3	0	0	0	0
4	1:160	0	1:640	0
5	1:200	1:200	1:640	0
Control	0	0	0	0

0 = no agglutination

It was further of interest to determine whether or not the phytoagglutinin had actually been removed from solution by the agglutinated red cells, and also whether or not any phyto-agglutinin had been removed by the tumor cells, which were not agglutinated. To determine this, the supernatant fluids of the tubes representing the end points in the agglutination reactions were assayed for the presence of remaining agglutinin. The supernatant from each series tested was divided into two portions, one to be titrated against rat erythrocytes and the other against chicken red blood cells. No agglutination of rat erythrocytes was obtained with the supernatant fluid following agglutination of rat erythrocytes by phytoagglutinins 2, 4, and 5. However, these supernates did agglutinate chicken erythrocytes in a dilution of 1:640. These results are given in Table III.

Using the supernates obtained from the first two dilutions in the tests with phytoagglutinin numbers 2, 4, and 5 on tumor cells, new agglutination tests with rat and chicken erythrocytes were also done. The rat erythrocytes were not agglutinated, indicating an adsorption of phytoagglutinins 2, 4, and 5 by the rat carcinoma cells, while chicken erythrocytes were again agglutinated in a dilution of 1:640. These results are tabulated in Table IV.

The results indicate that while agglutination occurs with rat erythrocytes and not with tumor cells, there is still evidence for the adsorption of the red blood cell agglutinin by both the red blood cells and tumor cells. On the other hand, the substance

TABLE III

Titers of Rat Red Blood Cell Adsorbed Supernates

Phyto. No. Supernatant	Titered With	Titer Obtained
2	Rat RBC	0
	Chicken RBC	1:640
4	Rat RBC	0
	Chicken RBC	1:640
5	Rat RBC	0
	Chicken RBC	1:640

0 = no agglutination

TABLE IV

Titers of Tumor Cell Adsorbed Supernates

Phyto. No. Supernatant	Titered With	Titer Obtained
2	Rat RBC	0
	Chicken RBC	1:640
2A	Rat RBC	0
	Chicken RBC	1:640
4	Rat RBC	0
	Chicken RBC	1:640
4A	Rat RBC	0
	Chicken RBC	1:640
5	Rat RBC	0
	Chicken RBC	1:640
5A	Rat RBC	0
	Chicken RBC	1:640

0 = no agglutination

responsible for chicken red blood cell agglutination was not found to be adsorbed by either rat erythrocytes or tumor cells.

Since the tumor cells obtained from the rat adsorbed three of the phytoagglutinins, it was desirable to ascertain whether this adsorption had any effect on the viability of the tumor cells. 3.0 ml of a 5% suspension of tumor cells was mixed with an equal volume of the stock solution of phytoagglutinin and placed on a shaker at room temperature for two hours, subsequent to which rats were inoculated subcutaneously with each mixture. Results as indicated by tumor growth are given in Table V. Phytoagglutinin No. 5 appears to have a marked inhibitory effect on tumor growth.

Phytoagglutinin No. 5 was adsorbed with chicken and rat erythrocytes in order to determine the inhibitory action of the adsorbed phytoagglutinin against tumor growth. 3.0 ml of a 5% tumor cell suspension was mixed in each instance with an equal volume of rat red blood cell adsorbed, chicken red blood cell adsorbed, and unadsorbed No. 5 phytoagglutinin, incubated at room temperature for two hours, and inoculated subcutaneously into rats. Results are given in Table VI.

It appears that the rat red blood cell agglutinin is associated with the tumor inhibiting fraction, since after removal of the rat red blood cell agglutinin by adsorption, a greater incidence of tumor growth is noted.

TABLE V

Tumor Growth Observed from Cells Treated with Unadsorbed Phytoagglutinins

Phyto No.	No. Rats	10 Days	15 Days	20 Days	25 Days
1	5	4/10 *	8/10	8/10	8/10
2	5	9/10	10/10	10/10	10/10
3	5	3/10	7/10	9/10	9/10
4	5	10/10	10/10	10/10	10/10
5	5	0/10	0/10	0/10	0/10
Control	5	10/10	10/10	10/10	10/10

* $\frac{\text{No. of tumor takes}}{\text{No. of sites inoculated}}$

TABLE VI

Tumor Growth Observed from Tumor Cells Treated with Adsorbed Phytoagglutinin No. 5

Phyto. No. 5	No. Rats	10 Days	15 Days	20 Days	25 Days
Chicken RBC Adsorbed	5	5/10 *	6/10	6/10	6/10
Rat RBC Adsorbed	5	5/10	10/10	9/10	9/10
Unadsorbed	5	1/10	1/10	1/10	1/10
Control	5	2/10	6/10	9/10	9/10

* $\frac{\text{No. of tumor takes}}{\text{No. of sites inoculated}}$

Since the in vitro protective action of No. 5 agglutinin gave such significant results against tumor growth in rats, it was also of interest to determine the in vivo effect. Immediately after the inoculation of tumor material into rats by the subcutaneous and intraperitoneal routes, 2.0 ml of No. 5 phytoagglutinin was injected intraperitoneally every day for a period of ten days. In another group of rats, the same type of treatment was begun five days after tumor inoculation, and was continued daily for six days. The results are given in Tables VII and VIII and indicate that in neither group did the treatment with phytoagglutinin have an inhibitory effect on tumor growth.

C. The Reaction of the PR8 Strain of Influenza Virus with Cells Derived from Normal and Malignant Tissues of the Rat.

The PR8 strain of influenza virus was selected for adsorption experiments because of its known action on the erythrocytes from various species. The receptor site for the virus particle on the chicken red cell surface, for example, has been characterized chemically by deBurgh and coworkers (63). Thus, a comparison of the tumor cell and other normal rat tissue cells as to their ability to adsorb this particular strain of influenza virus would be informative, indicating the existence of cell surface differences or similarities on the cells being considered.

The ability of cells derived from various normal tissues and of cells derived from tumor tissue to adsorb this virus was determined. Generally, a standard amount of virus in allantoic fluid was mixed with a known concentration of cells, and after

TABLE VII

Therapy of Rats with Phytoagglutinin No. 5 Beginning at Zero Days After Tumor Inoculation

Group No.	No. Rats	Route of Tumor Inoculation	Therapy	Route of Therapy	Tumor Growth 20-25 Days
1	5	2 sites Subcutaneously	Phyto. No. 5 Daily	2.0 ml Intraperitoneally 10 days	8/10 *
2	5	Intraperitoneally	Phyto. No. 5 Daily	2.0 ml Intraperitoneally 10 days	3/5
3	5	2 sites Subcutaneously	0.85% NaCl Daily	2.0 ml Intraperitoneally 10 days	7/10
4	5	Intraperitoneally	0.85% NaCl Daily	2.0 ml Intraperitoneally 10 days	4/5

* $\frac{\text{No. of tumor takes}}{\text{No. of sites inoculated}}$

TABLE VIII

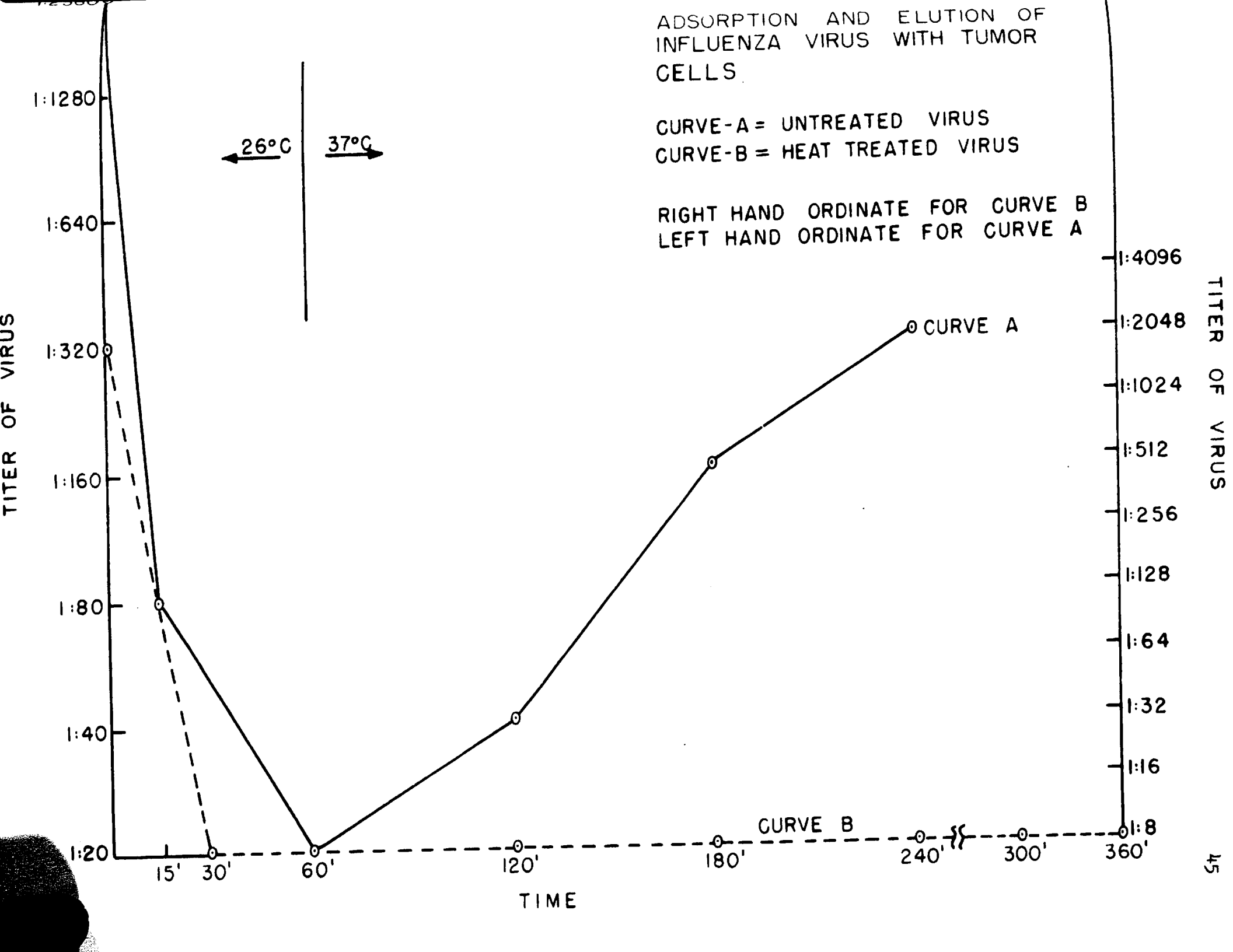
Therapy of Rats with Phytoagglutinin No. 5 Beginning 5 Days After Tumor Inoculation

Group No.	No. Rats	Route of Tumor Inoculation	Therapy	Route of Therapy	Tumor Growth 20-25 Days
1	5	2 sites Subcutaneously	Phyto. No. 5 Daily	2.0 ml Intraperitoneally 6 days	7/10 *
2	5	Intraperitoneally	Phyto No. 5 Daily	2.0 ml Intraperitoneally 6 days	4/5
3	5	2 sites Subcutaneously	0.85% NaCl Daily	2.0 ml Intraperitoneally 6 days	8/10
4	5	Intraperitoneally	0.85% NaCl Daily	2.0 ml Intraperitoneally 6 days	4/5

* $\frac{\text{No. of tumor takes}}{\text{No. of sites inoculated}}$

standing at room temperature for fixed time intervals, an aliquot of the mixture was removed, centrifuged, and the supernatant fluid was assayed for the presence of the virus with 0.25% chicken red blood cells. The presence or absence of the virus in the supernatant fluid as determined by the settled red cell pattern on the bottom of the reaction tube thus indicated whether or not adsorption had occurred between the virus and that particular cell. Elution of the virus from the cell was also measured in the same manner over a period of several hours.

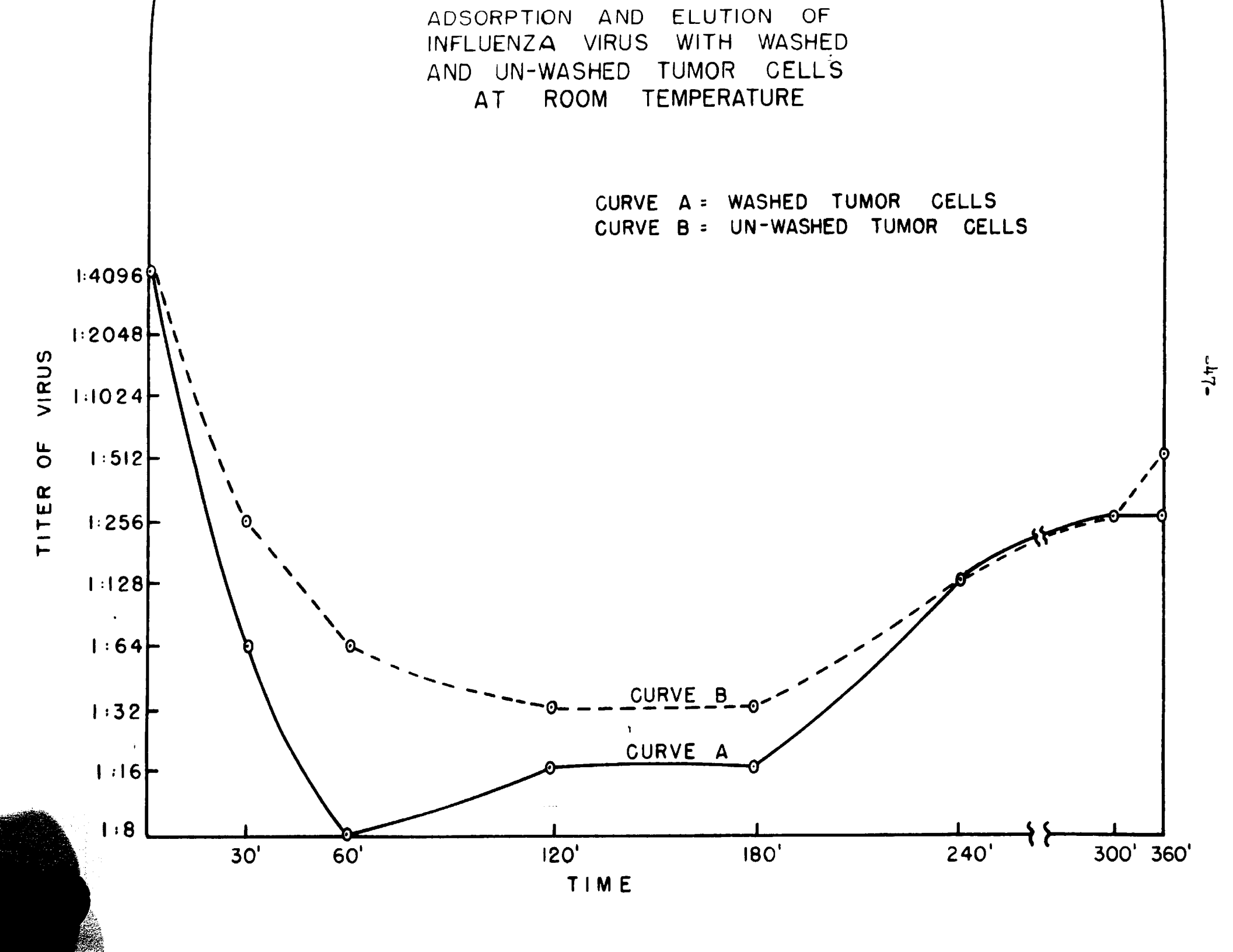
It was originally observed that 10.0 ml of a 1% suspension of tumor cells was capable of removing all of the detectable virus present in 1.0 ml of infected allantoic fluid in one hour at room temperature. After placing the mixture at 37°C, it was noted that a partial elution of the virus occurred. The adsorption-elution curve is given in Figure I. In further experiments, an attempt was made to determine the concentration of tumor cells which would remove all of a given amount of virus. Various concentrations of tumor cells were prepared: 20%, 15%, 10%, 5%, 2.5%, and 1%. To 7.5 ml of each dilution of cells was added 2.5 ml of virus in allantoic fluid to give a final virus dilution in the mixture of 1:4. At fixed intervals of time, aliquots of the mixture were withdrawn and the material assayed for the presence of virus. It was found that the tumor cells in concentrations of 5% and above removed all detectable virus from solution, while those concentrations below 5% did not remove all of the detectable virus from solution. In all subsequent virus-tumor adsorption experiments,

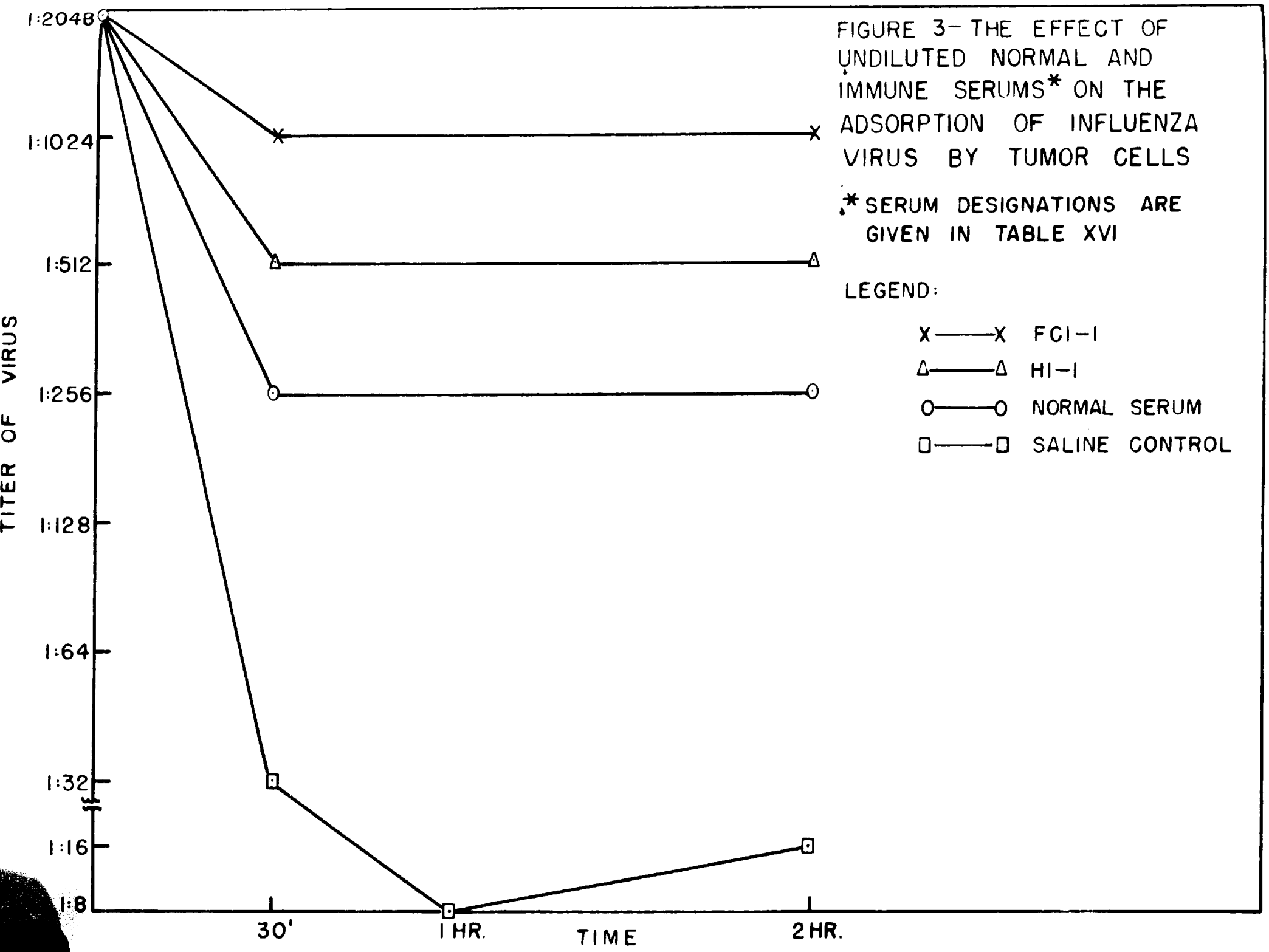

ADSORPTION AND ELUTION OF INFLUENZA VIRUS WITH TUMOR CELLS
CURVE-A = UNTREATED VIRUS
CURVE-B = HEAT TREATED VIRUS
RIGHT HAND ORDINATE FOR CURVE B
LEFT HAND ORDINATE FOR CURVE A
26°C
37°C
CURVE A
CURVE B
TITER OF VIRUS
1:1280
1:640
1:320
1:160
1:80
1:40
1:20
1:4096
1:2048
1:1024
1:512
1:256
1:128
1:64
1:32
1:16
1:8
15'
30'
60'
120'
180'
240'
300'
360'
TIME

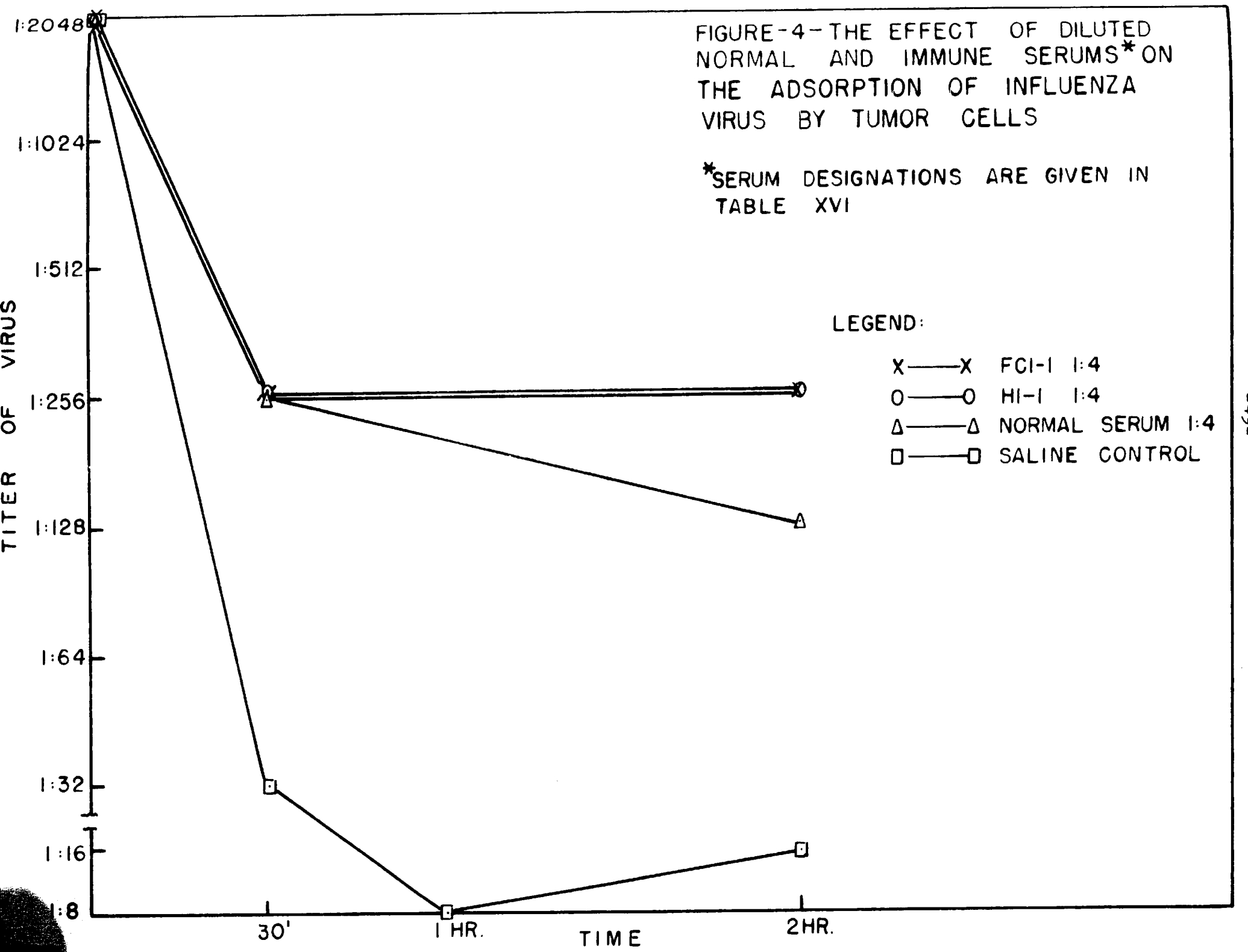
a 5% suspension of tumor cells was used, and a standard amount of virus was added to the tumor material in order to make a final virus dilution of 1:4. Using these proportions of tumor and virus, a typical curve as shown in Figure 2 was uniformly observed. If the tumor cells were not washed prior to use in such an experiment, less adsorption occurred initially, and oftentimes adsorption was not complete. This is also recorded in Figure 2.

An attempt was also made to determine the protective action of an antitumor serum against the adsorption of virus to tumor tissue. To 3.5 ml of a 10% suspension of tumor cells was added 3.5 ml of whole serum, and 3.5 ml of the same serum diluted 1:4 was added to a similar suspension of cells. All the samples were placed on a rotary shaker at 37°C for two hours. At the end of this time 1.0 ml of infected allantoic fluid was added to make a final virus dilution of 1:8. At intervals of thirty minutes and two hours aliquots of the mixture were withdrawn and assayed for the presence of virus. The results given in Figures 3 and 4 show some protection given by both antiserum and normal serum, with the former showing slightly more protective action than the latter, as contrasted to the adsorption of virus that occurs in saline solution. It is also noted that less protection occurs in the case of the diluted serums, as compared with the undiluted serums.

Hirst (50, 51) has shown that the effect of heat on influenza virus is such that the infectivity can be destroyed in the virus particle while the hemagglutinating activity either remains unaffected or is slightly lowered. The adsorption of such


ADSORPTION AND ELUTION OF INFLUENZA VIRUS WITH WASHED AND UN-WASHED TUMOR CELLS AT ROOM TEMPERATURE
CURVE A = WASHED TUMOR CELLS
CURVE B = UN-WASHED TUMOR CELLS
TITER OF VIRUS
1:4096
1:2048
1:1024
1:512
1:256
1:128
1:64
1:32
1:16
1:8
CURVE B
CURVE A
30'
60'
120'
180'
240'
300'
360'
TIME


1:2048
1:1024
1:512
1:256
1:128
1:64
1:32
1:16
1:8
TITER OF VIRUS
30'
1 HR.
2 HR.
TIME
FIGURE 3- THE EFFECT OF UNDILUTED NORMAL AND IMMUNE SERUMS* ON THE ADSORPTION OF INFLUENZA VIRUS BY TUMOR CELLS
* SERUM DESIGNATIONS ARE GIVEN IN TABLE XVI
LEGEND:
X——X FCI-1
Δ——Δ HI-1
O——O NORMAL SERUM
□——□ SALINE CONTROL


FIGURE-4-THE EFFECT OF DILUTED NORMAL AND IMMUNE SERUMS* ON THE ADSORPTION OF INFLUENZA VIRUS BY TUMOR CELLS
*SERUM DESIGNATIONS ARE GIVEN IN TABLE XVI
LEGEND:
X——X FCI-1 1:4
O——O HI-1 1:4
Δ——Δ NORMAL SERUM 1:4
□——□ SALINE CONTROL
TITER OF VIRUS
1:2048
1:1024
1:512
1:256
1:128
1:64
1:32
1:16
1:8
30'
1 HR.
2HR.
TIME

heat inactivated virus to tumor tissue was also determined. The virus was heated at 56°C for thirty minutes and was then added to a tumor cell suspension in fixed proportions. An assay of the supernatant from this mixture at definite intervals of time showed no detectable virus to be present, indicating no elution of the virus from the tumor cell. This observation is graphically presented in Figure 1, and table IX shows the effect of heat on the hemagglutinating activity of the virus. In order to establish loss of infectivity, a portion of the virus heated at 56°C for thirty minutes was diluted 1:100 in saline, and 0.25 ml of this dilution was inoculated into each of six 9 day old embryonated chicken eggs. Simultaneously, an equal amount of a similar dilution of unheated virus was inoculated into eggs of the same age, the results of which are given in Table X. It is apparent that the applied heat completely inactivated the infectivity of the virus.

It was of special interest to determine the effect of the adsorbed virus on the viability of the tumor cell. To 30 ml of a 5% suspension of tumor cells was added 10 ml of virus infected allantoic fluid. As a control, to another 30 ml of a 5% suspension of tumor cells was added 10 ml of normal allantoic fluid. At fixed time intervals, a sufficient amount of the mixture was withdrawn in each case to permit animal inoculation with the material, and, in addition, to permit testing of the supernatant for the presence of virus. After three hours at room temperature the mixtures were placed at 37°C to enhance elution. The adsorption-

TABLE IX

Influenza Hemagglutinin Titers of Heat Treated Virus

	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
Unheated	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
56°C for 15'	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++
56°C for 30'	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	0
56°C for 60'	++++	++++	++++	++++	++++	++++	++++	++++	0	0	0	0

0 = No Hemagglutination

TABLE X

Hemagglutination Titers of Allantoic Fluid From Embryonated Eggs Inoculated with Untreated and Heat Treated Virus

Heated Virus		Unheated Virus	
Egg No.	Titer	Egg No.	Titer
1	0	7	1:4096
2	0	8	1:4096
3	0	9	1:4096
4	0	10	1:4096
5	0	11	1:4096
6	0	12	1:4096

0 = No hemagglutination

elution curves obtained in these experiments were similar to that shown in Figure 2. Results of the animal tests are given in Table XI. All inoculations were given subcutaneously, two sites per animal. A similar experiment was carried out to determine the protection given by the virus against intraperitoneal tumor growth. Results of this are tabulated in Table XII. It is seen that there is more protection against tumor growth when the mixture is given subcutaneously than when it is given intraperitoneally.

The effect of inactivated influenza virus on the viability of the tumor cell is given in Table XIII. It is obvious from these results that protection against tumor growth is afforded only by virus which is in its infective state.

Therapy tests were also conducted in a group of rats which had been given subcutaneous injections of tumor tissue. Immediately after inoculation of tumor material, one group of animals was given 1.0 ml of PR8 virus infected allantoic fluid intraperitoneally, while a control group of rats received 1.0 ml of normal allantoic fluid by the same route. The therapy was continued daily for seven days. No attempt was made to recover virus from the grown tumors. Even though there was no apparent effect of the virus on the actual growth of the tumor, the rate of tumor growth seemed to be lessened and also the dimensions of the 20 day tumors were diminished in size from those of the controls. These results are summarized in Table XIV.

Normal rat tissues were also used experimentally in order to determine the comparative ability of influenza virus to adsorb to

TABLE XI

The Effect of Adsorbed Active Virus on the Ability of the Tumor Cell to Grow Subcutaneously

	Group	Inoculation Given At	No. Rats	Tumor Growth 20 Days	Controls No. Rats	Controls Tumor Growth 20 Days
Expt. No. 1	I	5'	5	0/10*		
	II	15'	5	1/10		
	III	30'	5	0/10		
	IV	60'	5	0/10	5	6/8 1 dead
	V	120'	5	0/6 2 dead		
	VI	180'	5	1/10	5	6/8 1 dead
	VII	240'	1**	0/2	5	10/10
Expt. No. 2	I	5'	5	0/10		
	II	240'	5	2/10	5	9/10
	III	300'	5	0/10	5	10/10

* $\frac{\text{No. of tumor takes}}{\text{No. of sites inoculated}}$

** Only one rat inoculated here due to insufficient inoculum.

TABLE XII

The Effect of Adsorbed Active Virus on the Ability of the Tumor Cell to Grow Intraperitoneally

Inoculation Given at	No. Rats	Tumor Growth 20 Days	Controls	
			No. Rats	Tumor Growth 20 Days
1 hr.	10	5/10 *	10	9/10

* $\frac{\text{No. of tumor takes}}{\text{No. of sites inoculated}}$

TABLE XIII

The Effect of Adsorbed Inactivated Virus on the Ability of the Tumor Cell to Grow Subcutaneously

Group	Inoculation Given At	No. Rats	Tumor Growth 20 Days	Control	
				No. Rats	Tumor Growth 20 Days
I	30'	5	8/10*	5	9/10
II	60'	5	10/10	5	8/10
III	180'	5	9/10	5	10/10
IV	240'	5	7/10	5	8/10
V	300'	5	5/10	5	6/10
VI	360'	5	1/10	5	0/10

* $\frac{\text{No. of tumor takes}}{\text{No. of sites inoculated}}$

TABLE XIV

Therapy of Rats with Influenza Virus Beginning at Zero Days After Tumor Inoculation

Group	No. Rats	Route of Tumor Inoculation	Therapy	Route of Therapy	Tumor Growth			
					10 Days	15 Days	20 Days	25 Days
I	5	2 sites Subcutaneously	Infected Allantoic Fluid (IAF) Daily	1.0 ml Intra-peritoneally 7 days	0/10*	3/10	6/10	8/10
II	5	2 sites Subcutaneously	Normal Allantoic Fluid (NAF) Daily	1.0 ml Intra-peritoneally 7 days	2/10	8/10	8/10	8/10

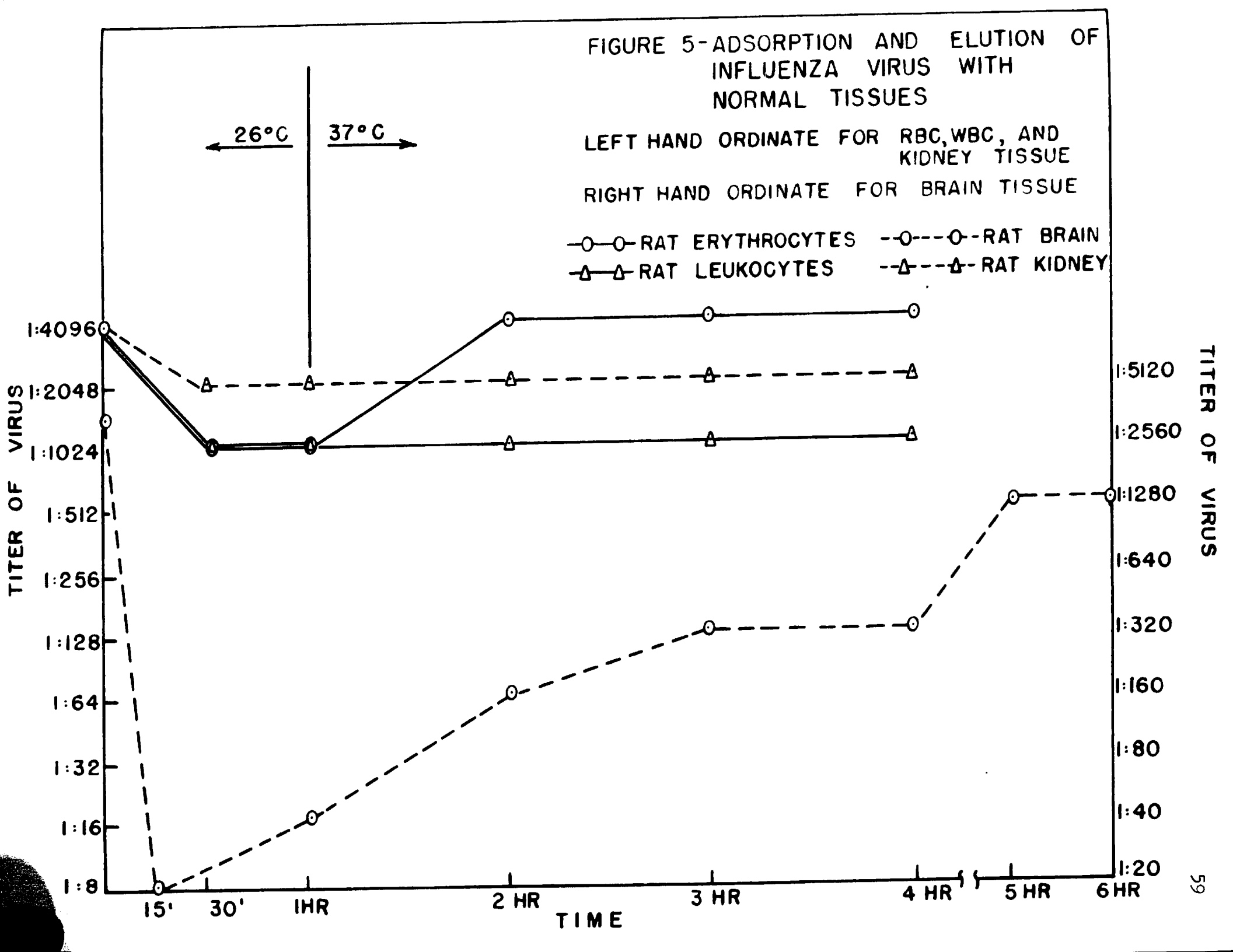
* $\frac{\text{No. of tumor takes}}{\text{No. of sites inoculated}}$

these tissues. The tissues used were normal rat brain, normal kidney, and rat leukocytes and erythrocytes.

To 10 ml of a 2.5% suspension of brain tissue was added 1.0 ml of infected allantoic fluid. At fixed intervals of time, samples of the mixture were withdrawn and the supernatant was examined for detectable virus. To each of 12 ml of a 5% suspension of rat leukocytes, kidney tissue, and red blood cells, was added 4.0 ml of infected allantoic fluid. At fixed time intervals aliquots of these mixtures were also examined for detectable virus to determine the degree of virus adsorption to these tissues. The adsorption-elution curves occurring between influenza virus and normal tissues are given in Figure 5. Of the tissues used, it is apparent that brain tissue is the only one that adsorbs influenza virus to any considerable degree, approximating the results of the adsorption curve obtained with tumor tissue. Rat erythrocytes, leukocytes and kidney tissue all indicate a partial adsorption of the virus, with the red cells giving the only evidence of elution.

D. The Reaction of Various Antiserums with Rat Erythrocytes, Leukocytes, and Tumor Cells.

It was attempted in this particular phase of the work to determine the serological relationships existing between the Flexner-Jobling cancer cell and the rat leukocyte and erythrocyte by the use of agglutination procedures and also by cross protection tests in animals. Antiserums to these cells were made in rabbits as described previously and these were used in all subsequent tests.


FIGURE 5-ADSORPTION AND ELUTION OF INFLUENZA VIRUS WITH NORMAL TISSUES
26°C
37°C
LEFT HAND ORDINATE FOR RBC, WBC, AND KIDNEY TISSUE
RIGHT HAND ORDINATE FOR BRAIN TISSUE
RAT ERYTHROCYTES
RAT LEUKOCYTES
RAT BRAIN
RAT KIDNEY
TITER OF VIRUS
1:4096
1:2048
1:1024
1:512
1:256
1:128
1:64
1:32
1:16
1:8
1:5120
1:2560
1:1280
1:640
1:320
1:160
1:80
1:40
1:20
15'
30'
1HR
2 HR
3 HR
4 HR
5 HR
6 HR
TIME

It was usually observed that tumor cells suspended in saline solution at room temperature would remain in stable suspension for 2-3 hours, but that after this time they would be seen to clump spontaneously. This spontaneous agglutination was also observed at 5°C but at a much slower rate. Further, when added to either normal or immune serums or dilutions thereof, tumor cells would agglutinate markedly and to a more rapid degree than those cells added to saline. Because of this, it was impossible to use the agglutination reaction with tumor cells. Protection tests in animals were used to give some indication of the anti-tumor potency of the immune serums developed in rabbits against tumor tissue.

In the case of rat leukocytes and erythrocytes, an optimal-proportion agglutination reaction was carried out to determine the optimal ratio of antigen to specific antibody to be used in the agglutinative procedure. The respective serums were first diluted with saline solution to give a final serum dilution of 1:10. Doubling serial dilutions of the serum were then made in saline and an equal volume of fixed concentrations of antigen was then added. The results given in Table XV indicate the optimal concentrations of antigen which were used. In all agglutination reactions, rat erythrocytes were used in a 0.75% suspension, whereas rat leukocytes were used in a concentration of 0.50%.

Cross agglutination reactions were carried out on a number of various antiserums against the three types of antigen. The numerical designation and types of these serums are given in Table XVI.

TABLE XV

Optimal Agglutination Titers of Rat Erythrocytes and Leukocytes With Their Respective Antiserums*

Concentration of Rat Erythrocytes	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240
2.0%	++++	++++	++++	++++	+++	0	0	0	0
1.0%	++++	++++	++++	++++	++++	+++	+++	0	0
0.75%	++++	++++	++++	++++	++++	++++	++++	+++	0
0.50%	++++	++++	++++	++++	++++	++++	+++	+++	0
0.25%	++++	++++	++++	++++	++++	++++	++++	+++	0
Concentration of Rat Leukocytes									
2.0%	++++	+++	+++	0	0	0	0	0	0
1.0%	++++	++++	+++	0	0	0	0	0	0
0.75%	++++	++++	+++	0	0	0	0	0	0
0.50%	++++	++++	+++	+++	0	0	0	0	0
0.25%	++++	++++	++++	+++	0	0	0	0	0

* Serums used in these tests:
No. R1-1 = Anti red blood cell
No. W4-2 = Anti white blood cell

0 = No agglutination

TABLE XVI

List of Various Antiserums Used in Serological Tests

Series No.	Number and Type of Immune Serums	Serum Designations
1	4 Anti Rat Red Blood Cell Serums	R1-1 R2-1 R3-1 R4-1
2	5 Anti Rat White Blood Cell Serums	W2-1 W3-1 W4-1 W3-2) Hyper- W4-2) immune
3	19 Anti Tumor Serums	
	A. 4 Tumor Free Cell	FC1-1 FC2-1 FC3-1 FC4-1
	B. 9 Tumor Homogenate	H1-1 H3-1 H4-1 H1-2) H2-2) H3-2) H4-2) Hyper-) immune H2-3) H3-3)
	C. 5 Tumor Pellet	R140A R141A R142A TP11A TP13A
	D. 1 Tumor Homogenate Developed in Rats	RTA-2

Cont'd. on following page

TABLE XVI Cont'd.

List of Various Antiserums Used in Serological Tests

Series No.	Number and Type of Immune Serum	Serum Designations
4	3 Anti PR8 Influenza Virus	PR-1 PR-2 PR-3
5	11 Normal Serums	
	A. Rabbit	RN-1 RN-2 RN-3 RN-4 TP11N TP13N R140 R141 R142
	B. Rat	RTN-2
	C. Rooster	CKN-1

Tables XVII and XVIII summarize the results of all the agglutination reactions carried out with absorbed and unabsorbed serums. It is seen that the leukocyte agglutinin in a number of antitumor serums is not removed either after the first or second absorption with tumor cells, nor is this agglutinin removed by rat erythrocytes. On the other hand, the antibody is removed by the absorption of these serums with white blood cells. These observations suggest the presence of the white blood cell or its antigen in the tumor immunizing preparations.

The red blood cell agglutinin present in the antitumor serums is removed by the addition of rat erythrocytes to these serums. There is also some suggestion of the removal of this red blood cell agglutinin by the white blood cells, but this erythrocyte agglutinating antibody is not removed by tumor material. Assuming all other conditions to be optimal, the results of these reactions suggest the absence of cross reactive antigens in the three types of cells studied.

The protection tests against tumor growth carried out in rats would not only indicate a certain measure of the potency of the antitumor serums, but they would also point to any cross protection afforded by antiserums to both rat leukocytes and erythrocytes.

Table XIX shows that both antitumor and normal serums not subjected to pretreatment with heat exhibit a certain measure of protection against tumor growth, and that these normal serums

TABLE XVII

Agglutination Titers of Rat Red Blood Cells and Rat White Blood Cells by Various Antiserums*

Serum No.	Unabsorbed Serums Titered With Rat RBC	Unabsorbed Serums Titered With Rat WBC	First Tumor Cell Absorption Titered With Rat RBC	First Tumor Cell Absorption Titered With Rat WBC	Second Tumor Cell Absorption Titered With Rat RBC	Second Tumor Cell Absorption Titered With Rat WBC	White Blood Cell Absorbed Titered With Rat RBC	White Blood Cell Absorbed Titered With Rat WBC	Red Blood Cell Absorbed Titered With Rat RBC	Red Blood Cell Absorbed Titered With Rat WBC
R1-1	1:2048	1:64								
R2-1	1:512	1:64								
R3-1	1:512	1:16	1:512	1:4	1:512	0	1:512	0	1:8	1:8
R4-1	1:512	1:64	1:512	1:32	1:512	1:8			1:4	1:32
W2-1	0	1:128								
W3-1	0	1:64	0	1:64						
W4-1	0	1:16	0	1:16						
W3-2	1:2	1:512	1:2	1:512	0	1:512	0	1:32	0	1:512
W4-2	1:4	1:256	1:4	1:256	1:4	1:256			0	1:256
R140-A	0	1:16								
R142-A	0	1:32								
TP11-A	1:8	1:64	1:8	1:64	1:8	1:64	0	1:4	0	1:64
TP13-A	1:8	1:4	1:8	0	1:8	0				1:4

* Serum designations are given in Table XVI

0 = No agglutination

TABLE XVIII

Agglutination Titers of Rat Red Blood Cells and Rat White Blood Cells by Various Antiserums*

Serum No.	Unabsorbed Serums Titered With Rat RBC	Unabsorbed Serums Titered With Rat WBC	First Tumor Cell Absorption Titered With Rat RBC	First Tumor Cell Absorption Titered With Rat WBC	Second Tumor Cell Absorption Titered With Rat RBC	Second Tumor Cell Absorption Titered With Rat WBC	White Blood Cell Absorbed Titered With Rat RBC	White Blood Cell Absorbed Titered With Rat WBC	Red Blood Cell Absorbed Titered With Rat RBC	Red Blood Cell Absorbed Titered With Rat WBC
FC1-1	0	1:32	0	1:32						
FC2-1	0	1:16	0	1:16						
FC3-1	0	1:32	0	1:32	0	1:32	0	0	0	1:32
FC4-1	0	1:16	0	1:16	0	1:16			0	1:16
H1-1	0	1:16								
H3-1	0	1:16	0	1:16						
H4-1	0	1:16								
H1-2	1:2	1:32	1:2	1:32						
H2-2	1:2	1:32	1:4	1:32						
H3-2	1:4	1:32	1:4	1:32	1:4	1:32	0	0	0	1:32
H4-2	1:64	1:64	1:64	1:64	1:64	1:64	1:16	1:4	0	1:64
H2-3	1:8	1:16	1:8	1:16	1:8	1:16			0	1:8
H3-3	1:8	1:8	0	1:8	0	1:8	0	0	0	1:8
TP11-N	0	0	0	0						
TP13-N	0	0	0	0	0	0	0	0	0	0

* Serum designations are given in Table XVI

0 = No agglutination

TABLE XIX

Protection Tests in Rats Against Subcutaneous Tumor Growth With Antiserum to Pellet Antigen*

Group	No. Rats	Serum Used**	Tumor Growth 20 Days
I	5	R-140	2/10***
II	5	R-140A	2/10
III	5	R-141	0/8
IV	5	R-141A	0/8
V	5	R-142	4/10
VI	5	R-142A	2/8
VII	5	Control	8/10

* All serums not inactivated in this experiment

** Serum designations are given in Table XVI

*** $\frac{\text{No. of tumor takes}}{\text{No. of sites inoculated}}$

afford a protection similar to that given by the serums developed against tumor tissue. When, however, these same serums of both types have been heat treated at 56°C for thirty minutes, it is indicated that a heat labile protective substance present in the normal serums is removed, whereas the heat stable cytotoxic property present in the antiserums is not removed. Evidence for this is shown in Table XX.

The protection given by rat leukocyte and erythrocyte serums as well as that protection given by antitumor serums is presented in Tables XXI and XXII. These data indicate that protection against tumor growth is afforded by antiserums prepared against both red blood cells and white blood cells, and that similar protection is given by only some of the antitumor serums.

E. Tumor Extract

Through the chemical fractionation of the chicken red blood cell, deBurgh (63) was able to isolate a component identified as polysaccharide in nature which was capable of inhibiting influenza virus hemagglutination in high titer. Much of the other recorded evidence points to the fact that the active agents responsible for viral hemagglutination inhibition are mucoprotein in nature (61, 62).

An aqueous tumor extract was prepared as outlined previously. With this material, hemagglutination inhibition tests were performed with influenza virus. Prior to conducting an experiment of this nature, it was necessary to convert the virus into the indicator phase. When the hemagglutinating action of a virus can

TABLE XX

Protection Tests in Rats Against Subcutaneous Tumor Growth With Antiserum to Tumor Pellet Antigen*

Group	No. Rats	Serum Used**	Tumor Growth 20 Days
I	5	R-140	7/10***
II	5	R-140A	2/10
III	5	R-142	8/10
IV	5	R-142A	2/10
V	5	TP11N	7/10
VI	5	TP11A	2/10
VII	5	TP13N	9/10
VIII	5	TP13A	3/10
IX	5	Control	9/10

* All serums heat inactivated at 56°C for 30 minutes

** Serum designations are given in Table XVI

*** $\frac{\text{No. of tumor takes}}{\text{No. of sites inoculated}}$

TABLE XXI

Protection Tests in Rats Against Subcutaneous Tumor Growth With Various Antiserums*

Group	No. Rats	Serum Used**	Tumor Growth 20 Days
I	5	RN-1	10/10***
II	5	R2-1	2/8
III	5	R3-1	4/10
IV	5	W3-1	0/10
V	5	H2-2	10/10
VI	5	H3-2	9/10
VII	5	H4-2	2/10
VIII	5	FC2-1	6/10
IX	5	FC3-1	8/10
X	5	Control	10/10

* All serums heat inactivated at 56°C for 30 minutes

** Serum designations are given in Table XVI

*** $\frac{\text{No. of tumor takes}}{\text{No. of sites inoculated}}$

TABLE XXII

Protection Tests in Rats Against Subcutaneous Tumor Growth With Various Antiserums*

Group	No. Rats	Serum Used**	Tumor Growth 20 Days
I	5	H2-3	6/10***
II	5	H3-3	1/10
III	5	W3-2	2/10
IV	5	W4-2	6/10
V	5	RTA-2	8/10
VI	5	RN-3	6/8
VII	5	RTN-2	7/8
VIII	5	Control	9/10

* All serums heat inactivated at 56°C for 30 minutes

** Serum designations are given in Table XVI

*** $\frac{\text{No. of tumor takes}}{\text{No. of sites inoculated}}$

be inhibited by a soluble inhibitor, it is said to be in the indicator phase (56). The conversion of active influenza virus into indicator virus can be accomplished simply by heating the virus in allantoic fluid at 56°C for thirty minutes. Virus thus heated loses virtually none of its hemagglutinating action, while the capacity of the virus to act as an enzyme has been lost. The antihemagglutinating titers of the aqueous tumor extract are given in Table XXIII. Parallel experiments with anti-influenza virus serums were also conducted. It is apparent that the tumor extracts give a slightly higher hemagglutination inhibition titer than those obtained with anti-virus serum.

Tumor extracts made in the manner indicated gave values of 1.15 to 1.20 mgm N per ml as determined by micro-Kjeldahl analysis.

Electrophoretic experiments were also conducted on the tumor extract. On a filter paper strip impregnated with 0.5 ml of the extract, two identifiable protein components were separated after six hours at 200V, with an approximate potential gradient of 4.25 V/cm. Tests conducted in an effort to elute these two components into saline to determine the antihemagglutinating activity of each individual component were negative. The electrophoretic patterns of the extract as developed on the Tiselius electrophoresis apparatus are given in Figure 6.

It is apparent that there are at least four major components in this extract, and possibly six. The electrophoretic mobility of each component is also given in Figure 5, as well as their relative amounts as computed on an areal basis with a planimeter.

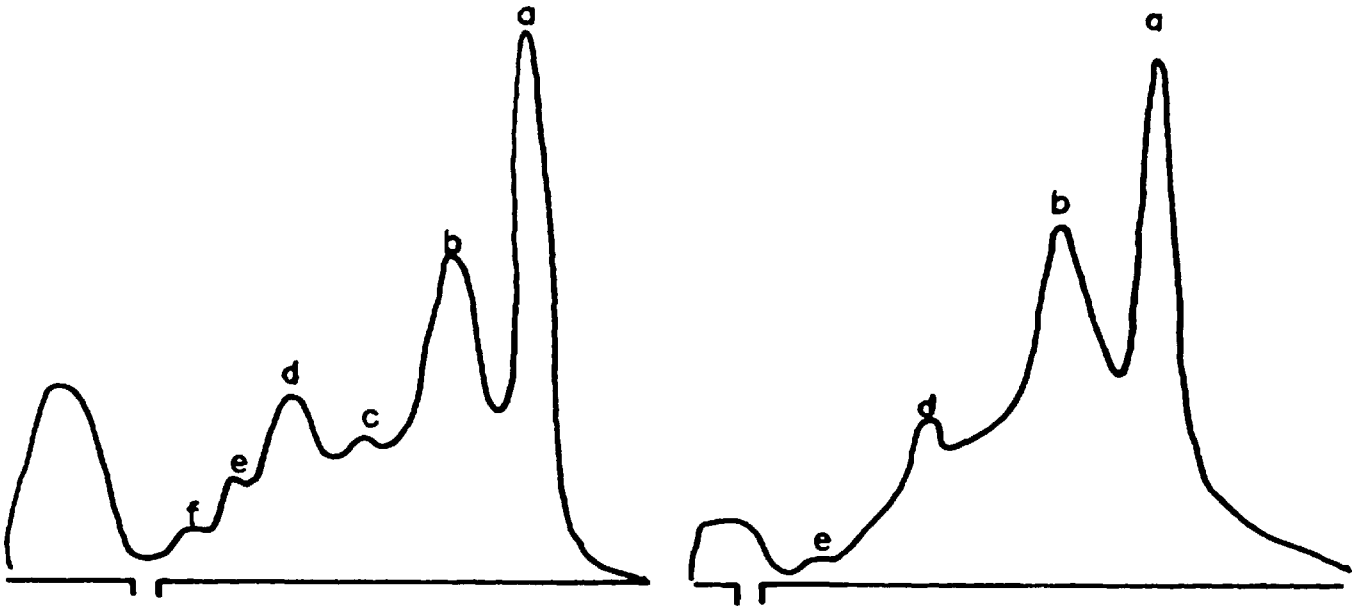
TABLE XXIII

Influenza Hemagglutination-Inhibition Tests With Dilutions of Tumor Extracts as Compared With Anti-Influenza Virus Serum

Materials Used	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096	1:8192
Extract 1 + Active Virus	±	±	±	±	±	±	±	++	++++	++++	++++	++++
Extract 1 + IAF* 56°C 15'	0	0	±	±	±	±	±	±	±	++	++++	++++
Extract 1 + IAF 56°C 30'	0	0	0	0	0	0	0	0	0	0	0	0
Extract 2 + IAF 56°C 30'	0	0	0	0	0	0	0	0	0	0	0	+++
Extract 2 + IAF 56°C 30'	0	0	0	0	0	0	0	0	0	0	0	0
Extract 3 + IAF 56°C 30'	0	0	0	0	0	0	0	0	0	0	0	+++
PR-1**	0	0	0	0	0	0	0	0	++	+++	++++	++++
PR-2	0	0	0	0	0	0	0	0	0	0	++++	++++
PR-3	0	0	0	0	0	0	0	0	0	++	++++	++++
CKN-1	0	±	±	++	+++	++++	++++	++++	++++	++++	++++	++++

Control Titers: Normal Allantoic Fluid = 0
IAF 56°C 30' = 1:2048
PSS = 0
Extract = 0

* Inactivated Allantoic Fluid
** Serum designations are given in Table XVI
0 = No hemagglutination



A. DESCENDING ARM B. ASCENDING ARM

FIGURE 6 — ELECTROPHORETIC PATTERNS OF AQUEOUS TUMOR EXTRACT, VERONAL BUFFER pH 8.6; MIGRATION TIME, 180 MINUTES

DESCENDING ARM:		ELECTROPHORETIC MOBILITY	AREA (%)
COMPONENT	a	5.04×10^{-5}	31.0
	b	3.96×10^{-5}	36.0
	c	2.87×10^{-5}	8.1
	d	2.06×10^{-5}	17.6
	e	1.32×10^{-5}	4.4
	f	$.65 \times 10^{-5}$	2.4

DISCUSSION

The observation by Loeb in 1901 (94, 95) that a tumor growth originating in a strain of Japanese waltzing mice could be successfully transferred to mice of the same strain but not to unrelated mice, opened a new approach to cancer research in animals. Geneticists in particular have been interested in the immunogenetics of tumor transplantation, and a considerable amount of work has been done resulting in the current point of view among these workers that the successful transplantation of both normal and neoplastic tissues is dependent on the simultaneous presence of dominant genes in both the implant and the host (96). It has been observed that if a tumor graft carries a dominant gene which functions in the development of a specific cellular antigen, and the recipient animal does not carry the identical gene in its tissues, the implant will evoke antibodies against itself to bring about its regression. However, if identical genes are present in both the tumor graft and the recipient animal's tissues, progressive tumor growth will occur (97). To illustrate the case in point, Gorer (31, 32) showed that a sarcoma arising in a pure line of albino mice could be transferred successfully to any other member of the same line but that it could not be transferred successfully to unrelated mice. Genetical studies by Gorer indicated that the growth or regression of the transplanted tumor was dependent on the presence of two dominant genes in the host. One of the genes was demonstrated to be identical with the gene determining the presence of a red blood cell antigen of these mice.

In a consideration of Gorer's work, the indication would be, then, that one should observe an antigenic similarity between tumor tissue and the normal tissues of susceptible animals. Gorer showed just such a similarity to exist, noting that an antigen identical to the one found in tumor tissue occurred in the erythrocytes of the susceptible animal. In the present study, the in vitro serological tests showed no such antigenic similarity to be present as far as the tumor cell and rat erythrocyte were concerned, although it must be remembered that an inbred line of animals was not used in this work. The antitumor serums developed in rabbits did exhibit varying low titers against rat red blood cells, but the presence of the red cell agglutinin in these serums was probably due to the presence of the contaminating red cell antigen in the immunizing preparations. Tumor tissue added to these antitumor serums did not remove the red cell agglutinin, as it most likely would have done had there been cross reactive antigens present in both types of cells. Further, red cell titers obtained with specific antiserums were not lowered to any extent after these serums had been absorbed with tumor tissue. Moreover, the red cell agglutinin present in the antitumor serums was removed by the absorption of these serums with rat erythrocytes.

No cross reactions were noted between the respective antigens and the antiserums to tumor tissue and rat leukocytes. Both the antitumor serums and anti white blood cell serums which were adsorbed with tumor tissue retained their agglutinating capacity for white blood cells, whereas this agglutinating antibody was

removed by the absorption of these serums with the specific antigen.

The presence of the white cell agglutinin in the antitumor serums is not surprising, since the incorporation of the leukocyte antigen in the tumor immunizing preparations was possible. Hirzfeld and his collaborators (24) observed cross reactions between human cancerous tissue and leukocytes, but they suggested that this observation might have been due to secondary leukocytic infiltration into the site of the tumor transplant. DaFano (98) noted that after the transplantation of mammary carcinoma into mice, lymphocytes and white blood cells accumulated around the graft and also became more numerous in the peripheral blood. Gorer (15), on the other hand, did find cross reactive antigens existing on the erythrocytes, white blood cells, and leukemic cells of the albino mouse. In addition, Steinberg (16) observed that human leukemic leukocytes were similar in antigenic structure to normal leukocytes.

The in vitro serological reactions between the red and white cells of the rat give a measure of suggestive evidence for the presence of cross reactive antigens in these two types of cells. While the erythrocytes were not capable of absorbing the white cell agglutinin present in the antitumor serums, in several instances the leukocyte was capable of absorbing the red cell agglutinin present in these same serums. Other than this evidence, however, related in vitro tests gave no further support to the presence of similar antigens on these cells. Neither the leukocyte nor the erythrocyte was able to remove the red cell agglutinin or the white

cell agglutinin, respectively, present in the specific antiserums to these cells. The fact that no demonstrable, clear-cut cross reactions were observed in vitro between the three types of cells studied does not preclude, however, the existence of similar antigens in tumor and other normal tissues, such as tissues of epithelial and mesothelial origin.

It is interesting to note here that Barrett's work (6) on the nature of the antigen in induced resistance to tumors indicates a relationship of the red cell antigen to the tumor antigen, in that it was repeatedly observed that the intact red blood cell from an animal of a different strain was able to induce an immunity to subsequent tumor inoculations in these same animals. This immunity could not be produced with leukocytes. On the other hand, Itami (4, 5) found that he was able to produce an immunity in mice with leukocytes prior to tumor inoculation, but was not able to do so with erythrocytes. These two observations indicate that in the animals used, no cross specificities were noted between the leukocytes and erythrocytes regarding induced resistance to tumors with these particular cells.

Hauschka in his recent review of cancer immunology (97) implies that the evidence currently on hand for the isolation of specific neoplastic antigens is generally unacceptable, and that it will continue to be so until the genetic "gap" which exists at the present time between the tumors used and the host in which they are grown is more carefully controlled. In other words, it is intimated that neoplasms, other than those of viral origin, which are propagated

even in so-called inbred lines of animals, do not elicit antibodies directed against themselves as malignancies, but do so merely as cells derived from genetically different individuals. On the other hand, Hauschka considers that the evidence supporting the existing quantitative differences between normal and neoplastic tissues is convincing.

There are also many instances cited in the data which are available that point to the common antigenic components contained by both normal and neoplastic cells. Red blood cells, white blood cells, and cells derived from many other normal tissues and their fractions, both adult and embryonic, are all capable of inducing a certain degree of immunity to subsequent tumor inoculation in a given animal. The fact that all these tissues and their derivatives are able to induce this protection against tumor growth in partially related lines is indicative of the cross antigenic relationships which exist between malignant and normal tissues. Malmgren et al (36) compared the microsome fractions of mammary tumor and hepatoma tissue serologically, and demonstrated the existence of qualitative differences between these two tissues. The differences, however, were attributed to organ specific antigens rather than to tumor specific antigens.

Maculla's study of mouse tissue components (28-30) has further revealed that antigenic relationships do exist between malignant tissues and normal tissues derived from adult and embryonic mice. Cross reactions were especially noted between tumor tissue and adult lung and spleen, but not with embryonic spleen. However, the

nucleoprotein fractions obtained from the tumors were found to be immunologically different from the nucleoprotein fractions of normal mouse organs.

The use of inbred lines of animals in cancer research undoubtedly has its place. The purpose of the present work, however, was to observe primarily the reactions of cells derived from normal and malignant tissues with some biologically active substances, and second, to note the effect of these substances on tumor growth. The nature of the problem, therefore, did not justify the use of an inbred line of animals, although the employment of such animals would have been ideal for the conduction of animal protection tests. The need of resorting to animal protection tests to assay the potency of an antitumor serum has become imperative due to the lack of other suitable and convenient methods for measuring the amount, specificity, and avidity of tumor antibody present in a given serum. All other known serological tests have been tried over a period of time by various workers interested in the development of a good procedure (39), but these attempts have not met with success. Difficulties are also encountered in the use of tissue culture, which, if overcome, would lead to the development of another valuable technique for assaying antitumor serums.

It was noted in the protection tests with various antiserums that the immune serums to the rat red and white cells gave a degree of protection against tumor growth which was similar to that observed with several of the antitumor serums. This might be interpreted as being due to the fact that red and white cells both

contain antigens which are common to the cancer cell. This fact however was not substantiated by the in vitro serological reactions. It was also noted in several instances that un-inactivated "normal" serums gave a degree of protection against tumor growth comparable to that protection given by the tumor immune serums. When these same "normal" serums had been heat inactivated at 56°C for thirty minutes, and subsequently again tested for their protective activity, no inhibitory action was noted, indicating that a heat labile substance was responsible for the protection previously observed. The tumor immune serums, however, retained this cytotoxic property after heat treatment.

It must be emphasized again that in the protection tests carried out in animals in this work, an inbred line of animals was not used, and therefore one cannot make a rigid quantitative statement concerning the amount of protection noted in the case of any antiserum. However, keeping in mind that the predetermined minimal dose of tumor material needed to produce the maximum number of tumor "takes" in rats was used in all experimental and control tests, one is justified, perhaps, in a comparison of the relative effects of any antiserum studied in inhibiting tumor growth with that amount of growth noted in controls. In all instances in which an antiserum afforded protection against tumor growth, a markedly greater growth was noted in the control animals. With this thought in mind, the assumption is then justified that the protecting ability of an antiserum is probably due to a peculiar cytotoxic property contained

in the serum. Whether this property is actually a specific antibody to tumor tissue is open to question.

No great differences in protective activity were noted between the antiserums obtained from the tumor homogenate and tumor free cell immunizing preparations, although it seems probable that the hyperimmune serums developed in the case of the tumor homogenate would be of greater value in protecting against tumor growth than the immune serums obtained from the same animals prior to the second and third immunizing periods. While several of the antiserums which were developed against tumor homogenate showed relatively no protective action against tumor growth, the hyperimmune serums obtained from these same animals did show an increased activity in protecting against tumor growth. It was also noted that the antiserums developed in rats which were naturally immune to tumor inoculations were completely ineffective in protecting against tumor growth. In other pilot experiments of the same nature, serums obtained from rats which were naturally refractile to tumor growth were incapable of producing any protection. This indicates that perhaps the immune state of these rats is not brought about primarily by an inhibitory property in the serum of the animals.

It is apparent that tumor tissue per se contains relatively weak antigenic constituents, whereas the cytoplasmic constituents and the cell wall fragments of the disintegrated cell exhibit an increased ability to bring about the production of tumor reactive antibodies. The serums made against the pellet fraction of tumor material seem to have given the best protection against tumor growth.

Dulaney and Arnesen (19) have shown the cytotoxic activity of antiserums developed against nuclei, mitochondria, and the microsome fractions obtained from normal and malignant spleens by sedimentation procedures. It was found that while the antiserums to both normal and leukemic spleen cell components exhibited some protective effect against the growth of leukemic cells, the immune serums developed against the nuclei and microsome fractions from the malignant spleens were much more damaging to leukemic cells in vitro than the antiserums to their normal counterparts.

A marked difference was noted between the Flexner-Jobling carcinoma cell and other cells derived from normal rat tissues regarding their respective abilities to adsorb the PR8 strain of influenza virus Type A. Red cells, white cells, and kidney tissue were found to adsorb the virus to a slight extent, but not to the degree that adsorption occurred with tumor cells. The only other tissue used that was also capable of adsorbing this virus was normal rat brain. It is interesting to note here that both brain tissue and the epithelium of the rat have a common origin, both being derived from the embryological ectodermal germ layer. The Flexner-Jobling carcinoma is a malignant growth arising from the epithelium of the albino stock rat.

It has been observed previously that no agglutination of rat erythrocytes occurs with influenza virus, with the exception of the WS strain of influenza type A (99). Nungester and coworkers (41), on the other hand, have noted that the PR8 strain also agglutinates rat erythrocytes. In the present work, however, it was found that

the technique of reading red cell agglutination according to the method described by Salk (92) gave unreliable results with rat erythrocytes since erratic patterns were obtained not only in the experimental tests, but also in the controls. Instead, by using chicken erythrocytes for assaying the supernatant fluid of a centrifuged mixture of rat red blood cells and influenza virus, it was found that some adsorption of the PR8 strain of this virus did occur with the rat red cell, and that elution of the adsorbed virus was complete at the end of two hours.

It was also observed that rat leukocytes and normal kidney tissue did not adsorb this particular strain of virus readily. However, a certain degree of adsorption was noted and apparently no elution of the virus occurred after the adsorption had taken place. In effect, then, it can be tentatively concluded that the erythrocyte, leukocyte, and kidney tissue of the rat do not have readily accessible cell surface receptors for the PR8 strain of influenza virus. On the other hand, it was found that tumor tissue as well as brain tissue behaved similarly in the adsorption and partial elution of the virus, which observation prevents any immediate deduction that the tumor cell surface is different from a related normal tissue cell surface as determined by the use of this virus.

The virus, however, was found to have a very pronounced effect on tumor growth. It was observed that when animals were inoculated with a mixture of active virus in allantoic fluid and viable tumor cells, almost complete inhibition of tumor growth occurred, in

contrast to a maximum amount of growth occurring in the control animals which had been inoculated with a mixture of normal allantoic fluid and viable tumor cells. This marked protection was noted in the case of subcutaneous tumor growth, in contrast to the lesser amount of protection which was noted in the case of intraperitoneal tumor growth. It was also observed that no inhibition of tumor growth occurred if the virus was heat inactivated prior to its addition to the viable tumor cell. In addition, in these latter cases no elution of the virus took place. It is therefore apparent that the adverse effect of the virus on the tumor is dependent on the presence of active virus. Moore (74) has also stated that virus which has been inactivated in any way by heat, U. V. rays, or formalin, is incapable of inhibiting tumor growth.

Given intraperitoneally as a therapeutic measure, beginning immediately after tumor inoculation and continuing daily for seven days, no prevention of growth against subcutaneous tumors was noted, although some inhibition was observed in noting that tumor growths were smaller in size from those in the control animals.

The early workers in the virus field, who considered actively growing tumors as possibly being a suitable medium for the propagation of viruses, were by nature of their approach to the problem not particularly concerned with the effect of viruses on tumor growth. Levaditi and Nicolau (68-70) were the first to observe that epitheliomas of mice and rats offered an excellent culture medium for the neurotropic strain of vaccinia virus. They soon observed, however, that it was not always possible to successfully

transfer the virus-carrying tumor to another susceptible animal.

More recently, workers have been interested in the oncolytic effect of viruses on tumors, with a view to finding a virus which would actively interfere with malignant growth.

Moore and her associates have been particularly interested in the effects of viruses on neoplastic growth. In one of her original publications (64), she made the observation that neither the PR8 strain of influenza virus nor the Herpes simplex virus was able to inhibit sarcoma 180 tumor growth in mice, under conditions in which fragments of tumor tissue were exposed to the viruses in vitro and then inoculated into animals. In the work reported in the present study, a very marked inhibitory action was noted on the Flexner-Jobling carcinoma with the PR8 strain of influenza virus. It must be kept in mind, however, that viruses appear to differ considerably and specifically in their respective abilities to exert a detrimental influence on different tumors. Oncolysis by viral action therefore is a specific affair, and hence it is difficult to say that a certain virus has no oncolytic properties, because of the possibility that it has not been tested against the proper tumor (74).

It was also noted by Moore (64) that the PR8 strain of influenza virus inoculated directly into sarcoma 180 had no effect on tumor growth. It was further reported by Kausche et al (67) that this same strain of virus did have an inhibitory action on the Ehrlich ascites tumor in mice, but that the inhibitory action was not complete. These workers also noted that in order for the

inhibitory effect to be produced, the virus had to be given simultaneously with or prior to tumor inoculation. Any virus that was given after tumor growth had become established did not induce a protective action. Pollard and Bussell (100) also noted that influenza A virus had a completely innocuous effect on two human and two rodent tumors in tissue culture.

The present study showed that an inhibition of tumor growth occurred even after the virus had eluted from the tumor cell. In Moore's experiments with Newcastle Disease virus and mouse sarcoma 180 cells (65), it was noted that after the virus-tumor combination had remained for 2-3 hours at room temperature, elution of the virus occurred, and, concomitant with elution, there was increased tumor growth. Since these workers were not able to demonstrate an actual multiplication of the virus in sarcoma 180 in either the animal or tissue cultures, they were led to believe that this inhibition of tumor growth was due to a surface phenomenon between the virus and the tumor cells.

In the case of the Flexner-Jobling carcinoma cell and influenza virus, no appreciable tumor growth occurred even after elution of the virus had taken place. Only in a few instances was it noted that tumor growth occurred after elution had taken place. It may thus be tentatively assumed that influenza virus exerts a damaging influence on the tumor cell, which effect apparently remains after the virus has eluted.

The fact that inactivated virus does not elute and does not exert a damaging influence on the tumor cell may indicate that the

portion of the cell surface concerned with virus adsorption is essential to tumor growth, and that through the enzymic action of the virus, which brings about the elution of the virus particle, this essential portion of the cell surface is disturbed in some way which leads to the inactivation of the tumor cell.

The mechanism of inhibition of tumor growth by viruses is not known. Various theories have been advanced by workers concerned with this problem. In cases in which actual viral multiplication is noted in either regressing or actively growing tumors, it is postulated that there is an actual entrance of the virus particle into the cell, and that the inhibitory action is due to a competitive antagonism between the virus and the tumor cell for an essential metabolite contained within the cell. This theory is however perhaps not applicable in the cases in which an adsorption and elution of the virus with the tumor cell is observed. Nevertheless, in instances in which only a partial elution of the virus occurs the question may be asked: where is the non-eluted virus? Does it remain on the surface of the tumor cell or does an actual entrance of some of the virus into the tumor cell occur? It is entirely possible that the latter may be the case with the PR8 virus and the Flexner-Jobling carcinoma cell and that a competitive process for a product which is essential to both the virus and the tumor cell is initiated upon the entrance of the virus into the cell, bringing about the inactivation of the latter. Other explanations that are offered as to the mechanism of action are that the virus may elaborate a toxin which is capable of producing severe damage to the cell (73),

and also that in the presence of virus a nonspecific stress is encountered in the cell which calls forth the production of adrenal steroids, which in turn bring about the desired therapeutic effect (76).

Since the tumor cell adsorbed the influenza virus particle in a demonstrable manner, it was of interest to procure an extract of tumor tissue in an effort to obtain a substance which would be capable of inhibiting influenza virus hemagglutination. A number of observations have been made on the inhibitory effects of red cell and tissue extracts, notable among which are the works of deBurgh et al (63), and of Green and Wooley (101), in which polysaccharides isolated from the chicken red cell and various other naturally occurring sources were found capable of inhibiting viral hemagglutination in very high titer. Friedewald, Miller and Whatley (102) have also worked with various tissue extracts to obtain substances which were capable of inhibiting viral hemagglutination. Using human materials, they noted that an inhibitory factor was found in human serum, and also in saline extracts of lung, liver, kidney and spleen. Similar results were obtained with serum and tissue extracts from normal rabbits and guinea pigs. It was also shown that the inhibitory substance obtained from red cells was identical with the receptor substance of the intact red cell which combines with virus to produce hemagglutination (102). Since these workers found a substance in tissue extracts which was capable of inhibiting viral hemagglutination, it is also possible that

this inhibitory substance is identical with the receptor site in these tissues for the viral particle.

In the present study it was found that an aqueous tumor extract was capable of inhibiting viral hemagglutination in a higher titer than that inhibition obtained with specific antibody to PR8 virus. This may be assumed to be due to the presence of receptor substances in the extract from the tumor itself.

Attempts to elute and isolate the two separable protein components which were obtained on a filter paper strip subjected to an electric current were not successful. An effort was made to elute these two components separately and to test each one for antihemagglutinating activity, but such tests resulted in negative data. It can be seen from the Tiselius patterns in Figure 6 that there are at least four major separable components present in the extract, and it is possible that either of the two components which were undetected by paper electrophoresis could be associated with the antihemagglutinating activity. Further it is also possible that the viral inhibition will occur only when each of the four components, or any combinations thereof, are present.

Much of the evidence available from other workers as regards the chemical nature of the inhibitory substances points to their being mucoproteins, although Burnet (56) cautions that none of these inhibitors, up to the present time at least, have been isolated as chemically and electrophoretically pure substances.

The use of phytoagglutinins in blood grouping is a recent development introduced by Boyd (42, 43). Aside from the practical importance of the use of such substances in blood grouping work, purification of these materials would yield important information as to the chemical groupings responsible for the blood group specific agglutinating activity. Chemical purification procedures have shown that the active agglutinating principle in the case of bean extracts is globulin in nature.

It was observed that even though rat erythrocytes were agglutinated by several of the plant extracts, tumor cells were not agglutinated. Subsequent tests revealed that though agglutination in the case of the latter did not occur, the rat erythrocyte agglutinin was removed from solution by the tumor cells. Animal protection tests showed that in the case of the phytoagglutinin extracted from the navy bean, there was definite interference with the viability of the tumor cell. When this extract was adsorbed with rat erythrocytes and then mixed again with tumor cells, growth once again occurred, indicating the tumor inhibiting fraction to be associated with the rat erythrocyte agglutinin. Therapy tests, on the other hand, did not show any prevention of tumor growth.

It is obvious from the tests which were conducted that the crude extract contains at least two different agglutinins, and possibly more. The isolation and purification of the active anti-tumor principle and its use in a more concentrated form may yield encouraging results, to the end that its future clinical application would be justified.

SUMMARY

The reactions of cells derived from normal and malignant tissues of the rat with certain phytohemagglutinins, the PR8 strain of influenza virus Type A, and specific antiserums has been determined. The effect of these biologicals on tumor growth was also noted.

The minimal amount of tumor material necessary to produce a maximum number of tumor "takes" in rats was determined.

Three of the five phytoagglutinins used were shown to be capable of agglutinating the rat red blood cell, but only one of the plant extracts was able to actively agglutinate rat white blood cells. Even though tumor cells were not actively agglutinated by any of the phytoagglutinins, it was shown that these cells removed the rat erythrocyte agglutinin from solution, but that they did not remove from solution the chicken erythrocyte agglutinin. The plant extract obtained from the white navy bean (Phaseolus vulgaris Linnaeus) was shown capable of markedly interfering with the viability of the tumor cell in vitro. Adsorption tests showed the tumor inhibiting fraction to be associated with the rat erythrocyte agglutinin. No effect on tumor growth was noted when this extract was given in vivo as a therapeutic measure.

Suspensions of both brain tissue and tumor tissue were shown to be capable of completely adsorbing a standard amount of influenza virus, while rat leukocytes, erythrocytes, and kidney tissue were shown to be incapable of doing so. Active influenza virus is also

capable of producing a maximum amount of protection against tumor growth, even after partial elution of the virus has occurred. Inactivated influenza virus does not protect against tumor growth, nor does this virus elute from the tumor cell. Given in vivo as a therapeutic measure, the virus is incapable of producing protective action against tumor growth.

An aqueous tumor extract was shown to be capable of inhibiting viral hemagglutination in high titer. Tiselius electrophoretic patterns indicated that the extract contained at least four and possibly six components. These components were characterized as to their electrophoretic mobilities and their relative concentrations.

No serological cross reactions were noted in vitro between the rat erythrocyte, leukocyte, and the tumor cell. Protection tests in animals indicated that the antiserums developed against a pellet fraction of tumor tissue were superior to those antiserums developed against tumor homogenates and free tumor cells. However, antiserums developed against both rat erythrocytes and leukocytes were also able to protect against tumor growth. Antiserums developed in and collected from rats which were naturally immune to tumor inoculations were not able to protect against tumor growth. It was also shown that "normal", pre-immunization serums which were not subjected to heat inactivation prior to use were capable of inactivating tumor cells in vitro. This cytotoxic property was shown to be due to a heat labile substance present in these serums.

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