INDUCTION OF ACTIVE IMMUNITY IN MICE

INDUCTION OF ACTIVE IMMUNE STATE BY MULTINUCLEATE TUMOR CELLS IN MICE

BY

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SCOPE AND CONTENTS:

Transplantable methylcholanthrene induced tumor was studied in relation to tumor immunity in its syngeneic hosts, A/Jax mice. The tumor was characterized by cytogenetic, histological and electron microscopic procedures. A technique was developed to establish a state of active immunity by immunizing animals with Sendai virus fused tumor cells. The specificity of immunoprotection was determined by the resistance to the challenges with viable tumor cells and the delayed hypersensitivity test. Adoptive transfer of anti-tumor immunity and cellular response of lymphoid cells from immunized mice were followed to examine the expression of cell-mediated reactions. Circulating antibody of the immune serum was demonstrated by immunofluorescent technique and the enhancing effect on the growth of the transplanted tumor. The relationship of these results to the current knowledge of tumor immunology was discussed.

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INTRODUCTION

Historical development

Progress in tumor immunity had to await the development and use of inbred mouse strains and a better understanding of the antigenic properties of the graft and the host. In the mean time, a vast, contradictary literature had accumulated and a great part of this confusion could be ascribed to the antigenic disparities existing among the experimental animals used by early investigators. The difficulties of research in tumor immunology before the realization of this fact have been described by many authors. The origin of tumor experimental research during the period before 1900 has been reviewed by Tyzzer (1916) and Triolo (1964). Other extensive reviews by Bittner (1935), Snell et al (1946) and Klein (1959) include the period before 1945 when the so-called "laws" of transplantation and immunological aspects of tumor transplantation were originally established.

The mouse systematic studies of tumor transplantation, according to Triolo's review, began with the work of Jensen (1903), Loeb (1902) and Ehrlich (1906) who were among the first to demonstrate that transplantation of a piece of tumor from one animal to another would result in progressive tumor growth in some cases. Loeb approached this problem by proposing and using animals of known ancestry (Bittner, 1935). A spontaneous tumor originating in the Japanese waltzing mouse strain was found to grow well in all members of the indigenous strain.

However, mice from a different albino stock were resistant and rejected the tumor. Leob drew attention to the variations existing among individuals of the same species that might affect the growth of the tumor. During the period of 1909 to 1916, Tyzzer and Little (1916) attempted to identify factors responsible for the differences in the susceptibility and resistance to transplantable tumors with respect to the genetic constitution of inbred, hybrid (between the strain of origin and the resistant strain) and backcross mice. The development of inbred strains began with Little's brother-to-sister mating of dilute brown mice and the inbreeding of other kinds of mice by Strong (1942). In 1922, Little and Strong formulated a genetic theory of transplantation. Briefly, it proposed that the outcome of transplantation depends on the degree of genetic similarity between graft tissue and the host. Through examination of hybrid and back-cross, they were able to demonstrate that susceptibility to transplanted tumors in mice depended on twelve to fourteen dominant genes (Little and Strong, 1924).

By 1935, further evidence in support of the genetic theory of tumor transplantation had established the importance of inbred animals in tumor research (Bittner, 1935). A brilliant series of investigations by Gorer, Snell and others demonstrated the importance of antigens in tumor and skin rejection. Gorer (1937, 1938, 1942) demonstrated the presence

of antigens located on erythrocytes and in the tissue of an inbred strain of mice. Tumor take or rejection by the animals was shown to be due to the presence or absence of alloantigens (isoantigens) on the transplanted tumor in relation to the host. Moreover, alloantibodies were induced in response to antigens found both in normal tissue and tumor grafts. Such antibodies could be measured by serological techniques using erythrocytes, leukemic cells or sarcoma cells, together with complement. Leukemic cells were neutralized by alloimmune serum resulting in failure of leukemic growth in mice. This suggested that alloantibodies play an important role in immunity to normal tissue and tumor grafts. In fact, the transplantation of tumors usually obeys the same rules that govern the grafts of the normal tissue. Lack of this knowledge has led to some perplexity in the past.

Gorer, Lyman and Snell (1948) found that the important histocompatibility gene 2 (H-2) locus was linked to the gene for the mouse tail deformity, "fused". This localized the histocompatibility gene to the ninth chromosome of the mouse. Since then, further efforts were made by Amos et al (1955a, 1955b), Gorer (1958, 1959), Snell (1953, 1954), Stimpfling 1965) and many others, who elucidated the complex H-2 locus which has many alleles. About 20 alleles are known at this locus, including combinations of at least 25 alloantigenic specificities (Snell, 1964; Snell and Stimpfling, 1966;

Stimpfling, 1969; Shreffler and Klein, 1970). Attempts have also been made to clarify the complexities of the weaker histocompatibility genes (Snell, 1958; Graff et al, 1966; Hildemann et al, 1970). H-2 has been considered the strongest histocompatibility gene, since the antigens associated with it have a strong capacity for inducing transplantation immunity when compared with other loci.

A better definition of histocompatibility barriers and genes governing the presence of alloantigens has also contributed to understanding of an interesting and paradoxical phenomenon. It is, so-called, "immunological enhancement" which suppresses rejection of the tumor graft. A major effort in studies of this phenomenon has been concentrated on locating the site of inhibition using the immune response to grafts. As knowledge regarding the sequential steps in immune responsiveness is still incomplete, attempts at localizing the immune blockage have been classified into three types of inhibition, namely, the afferent, central and efferent. Billingham, Brent and Medawar (1956) explained afferent inhibition as the capacity of antibodies to suppress the immune response by preventing the antigenic determinants from reaching the regional lymph nodes in an effective form; a central inhibition as one in which an antibody acts directly on cells engaged in antibody production; and an efferent inhibition is one which occurs at the target cell level by reaction between antibody and corresponding antigen receptors, leading to blockage of the latter.

Möller and Möller (1967) suggested peripheral block (afferent and/or efferent inhibitions) as the primary explanation for immunological enhancement. Immune cytotoxicity and immunological enhancement both depend on reactions between target cells and specific cellular or humoral antibodies. Serum antibodies kill the target cells in vitro by conventional complement-fixation reaction and cell death caused by participation of complement. Cellular antibodies seem to act by a different mechanism. Immune cells carrying a specific receptor attach to the target cells and cell death appears to be the result of close contact between these two types of cells; complement is not required for the reaction. Humoral antibodies were therefore considered to antagonize the effect of the cellular antibodies by competing for antigenic determinants of target cells. Through the investigations of allogeneic tumor-host systems, only IgG antibodies seem to promote immunological enhancement (Takasugi and Hildemann, 1969a and b). On the other hand, IgM antibodies appear to be involved in resistance to allogeneic tumors (Takasugi and Hildemann, 1969a). The relationship of IgM antibody cytotoxicity and tumor rejection, and action of IgG antibody in lowering both these effects in immunological enhancement has been interpreted in terms of feedback control mechanism. However, it has recently been suggested that IgG can manifest its cytotoxic action when the density of antigenic determinants on the surface of target cells is high (Linscott, 1970).

The introduction of graft antigens induced a lymphoid cell proliferation during the first week as detected in enlarged spleen and lymph nodes, as well as an increase of peripheral lymphoid cells (Woodruff and Symes, 1962; Bloom and Hildemann, 1970). As a final product of immunity, a population of antibodies arises. Little is known about the route of sensitization of the host to tumor cell antigens and equally scarce is information with regard to the actual mechanism of rejection. Gorer (1958) suggested at least three different effector systems in rejection. These include the cytotoxic effect of antibodies on leukemic cells, a rejection of solid tumor, such as sarcoma, by activated lymphoid cells, and the action of macrophages on ascites tumors. Rejection has been consistently associated with the adoptive transfer of immune lymphoid cells, but not necessarily with passive transfer of humoral antibodies. Nevertheless, this does not mean that serum antibodies are not involved in rejection.

Billingham, Brent and Medawar (1954) were the first to point out the similarity between transplantation immunity and delayed-type hypersensitivity. The reason for this proposition was, besides the histological features, the evidence showing the homograft sensitivity could be transferred to inbred normal "virgin" animals (those which had not been previously exposed to the grafts) by means of activated lymphoid cells from the regional nodes of actively sensitized animals. This transferrence of sensitivity or immunity by activated lymphoid

cells is known as adoptive transfer and was first domonstrated with respect to tumors by Mitchison (1954). Similar transfer has been shown by Billingham et al (1954) and Brent et al (1959) with orthotopic skin transplantation. Klein (1960), Koldovsky (1961) and Old et al (1962) transferred immunity to chemically induced tumors in inbred mice by administration of spleen and lymph nodes from immunized to non-immunized animals. A similar observation has been extended to tumor graft sensitivity in inbred guinea pigs (Oettegen et al, 1967; Oettegen et al, 1968). Although at one time it was a matter of dispute as to whether delayed hypersensitivity occurs in mice, it has in fact been established by several investigators using microbial pure protein antigens (Gray and Jenning, 1955; Crowle, 1959). Development of delayed hypersensitivity to chemically induced tumors has lately been demonstrated in guinea pigs, rats and mice (Kronman et al, 1969; Zbar et al, 1969; Wepsic et al, 1970; Wang, 1968; Halliday and Webb, 1969; Hoy and Nelson, 1969). In the mouse, delayed-type hypersensitivity is best demonstrated by injecting antigens in footpad. This has been done for a variety of antigens including allogenic tumors (Wang and Halliday, 1967) and chemically induced tumors of inbred mice (Halliday and Webb, 1969).

It has been assumed that tumor or homograft rejection is mediated through purely cellular processes, without incorporation of serum antibodies. This was based on the analogy

between delayed-type hypersensitivity and transplantation immunity, their lack of transfer by immune serum and reduced immunity associated with the phenomenon of immunological enhancement. Lately, however, this problem of the role played by humoral antibodies in the rejection of grafts has become the object of some controversy, part of which has been mentioned earlier in relation to immunological enhancement. At any rate, it seems safe to say that delayed-type hypersensitivity reactions, in addition to confirming the specific antigenicity of a tumor, provide a convenient means of detecting tumor antigens and also could be used as an assay during the extraction of these antigens, as suggested for guinea pig tumors (Churchill et al, 1968).

In light of recent development in immunobiology, small lymphocytes have finally been recognized as a class of cells with functional potentials. One of the most important functions of these cells appears to be necessary for expressing the state of specific immunity involving the differentiation of cells in at least two directions. One leads to the production of cells specializing in synthesis and release of humoral antibodies of the various immunoglobulin classes. The other leads to the production of specifically activated (sensitized) cells which are responsible for initiating the events recognized as cell-mediated immunity. The role of small lymphocytes in immune response has been reviewed extensively (Ford et al, 1965; Makinodan and Albright, 1966; Stone, 1967; Daniels

et al, 1968). By further investigations of Nowell's (1960) original observation it is now possible to study lymphocyte proliferation in vitro. This technique has allowed us to reconstruct and study the proliferative phase of the immune response in an independent system.

Lymphocytes may be induced to enter the mitotic cycle by various agents including plant extracts, phytohemagglutinin (PHA) (Gowans, 1962) and pokeweed (Farnes et al, 1964), anti-lymphocyte serum (Gräsbeck et al, 1964), anti-immunoglobulin serum (Sell and Gell, 1965), and bacterial products, streptolysin S (Hirschhorn et al, 1964) and staphyloccal culture filtrate (Ling et al, 1965). These are well known examples of nonspecific stimulators. The significance of the lymphocyte transformation induced with a nonspecific agent such as PHA is unclear. However, PHA induced blasts have morphological characteristics in common with cells transformed by specific antigens and with large pyroninophilic cells (LPC) that appear in certain cellular immune responses in vivo (Gowans, 1962). Therefore in vitro behavior of lymphocytes provides a model in which the in vivo immuno-proliferative response as well as differentiation can be studied. There is no convincing evidence that PHA acts as an antigen, though PHA induces an enormous stimulation of lymphocyte proliferation suggestive of the immune response. It becomes more reasonable to conclude that PHA operates primarily through gene activation (Pogo et al, 1966, 1967; Johnson and Rubin, 1970). Antigens may produce a similar effect, yet the stimulation may be **elicited** through a different mechanism.

Auto- and iso- immunity

Before 1953, the type of tumor transplantation immunity reported ocurred exclusively in situations where the host and transplanted tumor were of different genetical background and constitution. To develop anti-tumor immunity in syngeneic animals (i.e. colonies in which all members are genetically identical and will accept skin grafts from one another) against their strain-specific tumors as well as to develop immunity in autochthonous animals against their own tumors has been a central problem in experimental tumor research. The object of such investigations has apparently the prospect of tumor therapy, hoping that tumor immunity could be induced by methods used in microbial immunity. However, it became clear gradually that the immunity, which could be induced against a transplanted tumor, did not produce immunity to spontaneous tumors. In light of the development of immunogenetics, an improved understanding of the genetic determination of histocompatibility, which is common to both neoplastic and normal tissues, was realized. Although animals could be immunized against tumors from genetically different animals, it seemed apparently not possible to immunize them against their own tumors when no known histocompatibility differences existed. Nevertheless, attempts to demonstrate tumor specific immunity have continued. Efforts in this direction have been disappointing, and some cases where positive results were claimed, there was disagreement with regard to interpretation.

Foley (1953) reported that removal of transplanted tumors,

recently induced by methylcholanthrene (MC), resulted in some resistance to subsequent challenge with the same tumor. However, when a "spontaneous" adenocarcinoma was grafted to other syngeneic mice and thereafter surgically removed, no immunity could be demonstrated to the same tumor. Prehn and Main (1957) similarly found that 12 out of 14 MC-fibrosarcomas could produce immunity to each tumor in inbred animals. Yet, seven spontaneous fibrosarcomas of the same histological appearance as the induced neoplasms, did not induce immunity. The same authors also demonstrated that no immunity to iso-antigens of tumor cells was induced by transplantation. The possibility of residual heterozygosity in the experimental mice has been excluded by their findings; (i) pretreatment with normal tissues from the original mouse did not produce tumor resistance; (ii) skin from the primary tumor host was not rejected when grafted to syngeneic mice pretreated with the tumor; and (iii) mice of the strain used accepted intrastrain skin grafts. These results strongly suggested that each of the MC-induced tumors had specific antigens not present in normal mouse tissues, while the spontaneous tumors possessed no such detectable antigens.

The results of Foley and of Prehn and Main were confirmed by Revesz (1960), Klein et al (1969) and Old et al (1962). Among some of these experiments, a different immunizing technique was employed. X-irradiated tumor cells were injected subcutaneously

and challenge doses consisted of graded numbers of viable tumor cells. In the investigation of Revesz, before giving the tumor graft the mice were injected twice with irradiated cells from the same tumor taken from its first and second transfer in other syngeneic hosts. While the tumor took in 56% of the control mice, it developed only in 15% of the treated mice. Pretreatment with normal tissues did not affect the outcome of tumor transplantation nor was the persistance of skin grafts influenced by injection of irradiated sarcoma cells. However, no clear-cut resistance could be demonstrated against mammary adenocarcinomas originating spontaneously in inbreeding females or against spontaneous lymphomas. Klein et al (1960) showed that in 19 out of 22 tumors, 5 to 1000 times more cells were necessary for progressive growth in the treated animals than in untreated controls.

In an investigation by Hirsch et al (1958), an attempt was made to immunize inbred mice by injecting them with the first transplant-generation of spontaneous adenocarcinoma cells. The mice were then challenged with the second transplant-generation of the same tumor after removal of the first tumor. No difference in time of appearance of tumor or in mortality rate between experimental and control groups were found. Nevertheless, the treated mice showed a small but significant increased survival time compared with the controls. These results might be considered as evidence that inbred mice developed weak immunity against spontaneous tumor due to their possession of weak tumor specific transplantation antigens. In fact, some tumors, considered as spontaneous in

origin, are now known to be virus induced, such as mammary adenocarcinomas. When appropriately studied in virus-free mouse strain, these tumors were shown to induce tumor specific transplantation immunity (Riggins and Pilch, 1964). Most of the high tumor incidence strains of inbred mice are carrying the virus which was passed from mother to young. The young developed a tolerance by early exposure to the virus (vertical transmission) and the animals showed absence of immunological reactivity, but not because tumor specific transplantation antigens (TSTA's) were lacking.

The question whether it would also be possible to induce immunity against MC-sarcoma in the primary autochthonous host has been studied by Klein et al (1960). The tumor-bearing leg was amputated, and heavily irradiated cells from the tumor injected back into the same mouse; two additional immunizations were given of irradiated cells from the two first transfers of the same tumor in the syngeneic hosts. Twelve of the 16 primary tumor hosts tested showed resistance to their own tumors. In this case the possibility of heterozygosity in animals was completely ruled out, since the tumor challenge under this given condition was an autograft. The results also showed that animals bearing tumors are not incapable of protecting themselves against their own autochthonous tumors.

The existence of tumor specific antigens in MC-induced tumors is well documented (Klein et al, 1960; Old et al, 1962;

Prehn, 1963; Old and Boyse, 1964; Sjörgen, 1965; Klein, 1966). One advantage of using chemically induced tumors in the experimental tumor research is that each tumor appeared to be antigenically different from other tumors, despite the fact that they are induced in the same animals or in mice of the same genotype (Klein and Klein, 1962; Old et al, 1962). However, Prehn (1963) has pointed out two pairs of tumors which showed cross reaction, namely, animals immunized with one tumor of the pair produced resistance against itself as well as against the other tumor. The specificity of the immune reactions of this type has been questioned, since it has been demonstrated that treatment of mice with tumor or normal tissues sometimes induces a nonspecific stimulation of the primary immune response against a subsequent antigenic tumor challenge (Klein and Klein, 1962). This nonspecific effect, in contrast to a tumor specific stimulation, could be eliminated by a low dose of X-irradiation (350-400 rads) of the treated animals, 24 hours prior to tumor challenge. This technique has also been used to detect minimal resistance to chemically induced tumors (Klein et al, 1960; Sjögren, 1964; Baldwin et al, 1967a).

MC-induced tumors, as the prototype of chemically induced neoplasms, have been investigated extensively, since these tumors possess relatively strong tumor-specific antigens. Tumors induced by other carcinogens have also been tested in similar experiments. For example, tumors induced by 3,4 benzpyrene (Feldman et al, 1963; Old et al, 1962), diben (a,h) anthracene

(Prehn, 1960) and aminoazo dye (Baldwin et al, 1967a, 1967b, 1969).

With knowledge concerning the principles controlling the identification and response of tumor specific antigens of transplantation type in animals, it is always exciting and important to develop a technique of establishing a state of active immunity in syngeneic host animals. In addition to the application of conventional tumor immunizing techniques as previously described, immunization to certain tumors also has been achieved with tumor cells treated with garlic extract (Fujiwara and Natata, 1967), iodoacetate (Apffel et al, 1966), nitrogen mustard N-oxide (Ishidate, 1967) or neuraminidase (Currie and Bagshawe, 1967, 1968a, 1968b, 1969; Bagshawe and Currie, 1968). The rationale of inducing immunity with these various treated tumor cells seemed to obtain attenuated cells which retained or enhanced their tumor antigenicity.

Background of the present study

It is believed that a barrier of sialomucin layers in the zona pellucida of blastocyst and in the placenta prevent the mother's recognition of the conceptus as non-self (Billingham, 1964; Billingron, 1967; Bagshawe, 1967; James, 1969). A similar barrier which prevents recognition and rejection of the conceptus (alloantigens) also exists on the cell surfaces of malignant tumors on which TSTA's are thought to be masked (Currie and Bagshawe, 1967). Tetraploidy of the conceptuses has been shown to be lethal and the conceptuses regularly aborted in mouse (Beatty, 1957) and in man (Carr, 1970). It seems possible that these conceptuses with higher ploidy of chromosomes were rejected by the mother and aborted on an immunological basis, as these conceptuses have not been found to develop to term. An attempt was made to evaluate the response of the body to the tetraploid cells by using MC-induced tumor and its syngeneic host (mouse).

In this connection, colchicine was used to induce tetraploid tumor cells in early experiments, but it was not regularly successful. Fusion activity of Sendai virus in cells has come to our attention (Okada, 1962a, 1962b; Harris et al, 1966). The virus could induce near-diploid MC tumor cells to binucleate (potentially tetraploid) and other higher multinucleate (polyploid) cells. Ultraviolet light was used to inactivate virus infectivity

without affecting the property of cell fusion, since mouse cells are permissive to Sendai virus infection (Yerganian and Nell, 1966). The subject of main concern in this thesis is a technique involving the injection of multinucleate cells fused by treatment with Sendai virus. Active immunity was established using multinucleate MC tumor cells in A/Jax mice. The degree of immunogenicity in this system was tested and compared with that induced by neuraminidase treated or gamma (γ) irradiated tumor cells. Moreover, immune state of the immunized animals were further assessed by delayed hypersensitivity test and adoptive transfer of anti-tumor immunity with lymphoid cells from immunized animals. An attempt was also made to relate *in vitro* information with and without presence of PHA to *in vivo* immunoproliferative response of lymphoid cells in immunized hosts using tritiated thymidine incorporation assay.

MATERIALS AND METHODS

I. Tumors and Host Strain

The first tumor, designated as MC, was obtained from Dr. R.C. Buck, University of Western Ontario, London, Ontario. This tumor was induced by subcutaneous injection of 3-methylcholanthrene* in a strain A/Jax male mouse in 1961. Since then it has been carried in its indiginous hosts by intramuscular injection of a cell suspension. The MC tumor was histologically characterized as a sarcoma.

The second tumor, designated as SP, was used in some experiments as a control. This tumor arose spontaneously on the neck of an A/Jax male mouse which was immune to MC tumor at the time of occurrence. The histological features of the primary SP tumor were of parotic salivary gland myoepithelioma, as it appeared that some areas of spindle shaped cells were present in bundles and a pseudoacinar pattern was shown in some other areas. Encapsulation of the tumor was noted macroscopically and microscopically.

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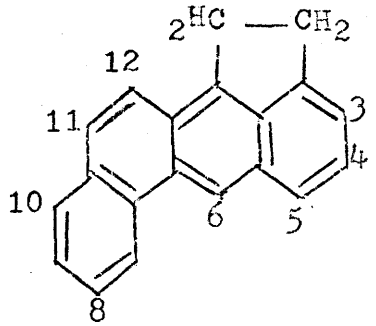
* The nomenclature of methylcholanthrene has been changed from 20-methyl- to 3-methyl-cholanthrene in accordance with IUPAC nomenclature rule (A-23.1). The numbering and structure orientation of cholanthrene are given below:



Nomenclature of Organic Chemistry, (1957) Butterworths Scientific Publications, London, England.

Both MC and SP tumors were transplantable in A/Jax mice, either male or female. The A/Jax mice were obtained only from the Jackson Memorial Laboratory, Bar Harbor, Maine. The diet consisted of Purine Chow Breeder Pellets. For use in the experiments, the mice were randomly distributed in polyethene cages in groups of five. To avoid any immune reactions associated with sex determined antigens, only male mice were used throughout the experiments as both tumors were originally derived from males.

Tumors were removed aseptically from the mice killed by cervical dislocation. Single cell suspensions were prepared by teasing the tissue gently in a Petri dish containing Hanks' Balanced Salt Solution (HBSS) (Difco Laboratories, Detroit, Mich.). Tumors were minced using scissors. The chopped tumor suspension was then sucked into one ml. syringe through a 23 gauge needle when the fragments were largely dispersed. The suspension was processed, counted and diluted to a desired cell concentration. One to 6 million tumor cells were injected about every two weeks into 2 to 3 virgin male mice for maintaining the tumor lines.

II. Histological Techniques

Pieces of tissue were fixed overnight in 10% neutral buffered formalin or Davidson's fixative (formalin, 20 parts; 95% ethanol, 35 parts; glacial acetic acid, 10 parts; and distilled water, 35 parts). After fixation was complete, the

tissues were then embedded, sectioned and stained with hematoxylin and eosin in a routine manner carried out in this laboratory.

III. Electron Microscopy

1. Reagents

(a) Phosphate Buffer: Solution A=7.1 gm. $NaHPO_4$ in 500 ml. distilled water. Solution B=6.8 gm. KH_2PO_4 in 500 ml. distilled water. Before use, 7 parts of solution A were mixed with 3 parts of solution B to give a solution of 0.1 M, pH 7.2.

(b) Veronal Buffer: 1.5 gm. of sodium veronal (barbital) and 1.0 gm. of sodium acetate were dissolved in 50 ml. distilled water.

(c) Osmic Acid: 2% buffered osmic acid (British Drug Houses Ltd., Poole, England) was made by adding 30 ml. distilled water, 10 ml. of 0.1 N HCl with 1 gm. of osmium tetroxide and 10 ml. veronal buffer. After 24 hours at room temperature, 2.25 gm. of sucrose was added to the solution.

(d) Glutaraldehyde: 3.5% glutaraldehyde solution was prepared by mixing 7 ml. of 25% glutaraldehyde (Eastman Organic Chemicals, Rochester, New York) with 43 ml. of phosphate buffer.

(e) Epon: Solution A was prepared by mixing 62 ml. of Epon 812 (Shell Chemical Company, New York) and 100 ml. of Dodecenyl succinic anhydride (DDSA) (E.V. Roberts and Associates Inc., Cuver, California). Solution B was made by mixing 100 ml. of Epon 812 and 89 ml. of nadic methyl anhydride (NMA)

(E.V. Roberts and Associates Inc.). Prior to embedding, solution A, solution B and accelerator, 2,4,6-tri-di-methyl phenol (DMP) were mixed in the proportions 49%: 49%: 2%.

(f) Uranyl Acetate: 6% uranyl acetate (British Drug Houses Ltd., Poole, England) was made up in absolute methanol. The solution was cooled and filtered through Whatman No. 1 filter paper before use.

(g) Lead Citrate: 0.35% lead citrate was prepared by adding 0.1 ml. of 10N NaOH and 0.35 gm. of lead citrate to 100 ml. of distilled water.

2. Fixation, embedding, sectioning and examination

Thin tumor fragments were rinsed with cold HBSS, then prefixed in 3.5% glutaraldehyde in phosphate buffer of pH 7.2 for 20 minutes. The glutaraldehyde solution was discarded and replaced with 2% osmium tetroxide in veronal buffer of pH 7.4. This post-fixation process was continued for 40 to 60 minutes. The specimens were then dehydrated in 70%, 95% and absolute ethanol for 15 minutes each in an ice bath, then passed through two changes in propylene oxide for 15 minutes at room temperature and embedded in Epon 812.

The blocks were incubated at 60°C for 24 hours. Following trimming, the blocks were cut with a Reichert ultramicrotome equipped with a glass knife. Ultrathin sections on water reflecting grey or silver were selected, mounted on the dull side of 300 mesh copper grids (Ernest Fullam Company, Shenectady, New York) and stained with 6% uranyl acetate in methanol for 15 minutes at room temperature, then rinsed in

three changes of absolute methanol. The sections were double stained with 0.35% lead citrate for 10 seconds and rinsed in two changes of water. The above mentioned staining and rinsing processes were performed by placing the section side of the grids down on a drop of stain or solution which was previously dropped in the flat surface of a dental wax plate. Observations and electron micrographs of sections were made in a Philips EM 300 with a 60-Kv beam or a RCA 3H with 50-Kv beam electron microscope.

IV. Diffusion Chamber Culture Technique and Chromosome Preparation

1. Diffusion Chamber Culture Technique

All cultures for chromosome preparations were made from short term cultures in vivo by a modification of the diffusion chamber technique of Gilman and Basrur (1968). A cell suspension containing approximately 1-3 x 10^6 cells, or small fragments of tumors or embryonic tissues in HBSS were introduced into the open side of a sterile diffusion chamber whose other side had previously been sealed with membrane. The open side was then sealed by membrane with MF cement in the assembly tool (Millipore Ltd., Montreal). The complete chambers were submerged in prewarmed HBSS until implanted by surgical technique into the peritoneal cavity of each recipient mouse and the animals were kept in cages for 3-4 days.

2. Chromosome Preparations

(1) Reagents

(a) Colchicine Solution: The stock solution was prepared from colchicine powder, $C_{22}H_{25}NO_6$ (alkaloid, U.S.P., Fisher Scientific Co., New York) to 1 mgm. per ml. in distilled water and stored at 4°C for no longer than two months before use.

(b) Hypotonic Solution: Sodium citrate was used to swell the cells prior to fixation. Both for embryonic cells and tumor cells, 0.9% sodium citrate in distilled water was used.

(c) Fixative: The harvested cells were fixed with acetic alcohol, freshly prepared by mixing one volume of glacial acetic acid with three volumes of absolute ethanol. Subsequently, 45% acetic acid was used to rinse the acetic alcohol from the fixed cells prior to staining.

(d) Carbol Fuchsin Stain: Staining solution was prepared by mixing 45 ml. stock solution B with 6 ml. glacial acetic acid and 6 ml. 37% formaldehyde. Stock solution B was made up by 10 ml. stock solution A (Basic fuchsin [CF-41: Coleman and Bell], 30 gm; 70% ethanol, 100 ml) and 90 ml. 5% phenol distilled water. The solution was cooled and filtered through Whatman No. 1 filter paper before use.

(2) Techniques

To arrest the mitosis of the cells in the chambers the recipient animals were injected intraperitoneally with colchicine at the rate of 1 gamma per 1 gm. of body weight approximately three hours before chamber removal. Care was taken not to puncture the chambers at injection. After the

chamber was removed from the animal, membrane from one side of the chamber was cut open and cell suspension was transferred into a centrifuge tube. To ensure that most cells were removed, the inside of the chamber was then flushed with HBSS and the suspension was pooled.

The subsequent procedure of chromosome preparation was a modification of the technique for leucocytes described by Carr and Walker (1961). HBSS was removed by centrifugation at 1,000 rpm in a clinical centrifuge for 10 minutes. The cells in the tube were treated with prewarmed (37°C) 0.9% sodium citrate for 10 minutes at 37°C. After centrifugation and removal of the supernatant hypotonic solution, the pellet of cells was fixed with chilled fresh acetic alcohol for 10 minutes and rinsed with 45% acetic acid in water. A small amount of 45% acetic acid was left in the tube making a moderately dense suspension. One or two drops of cell suspension were placed in a precleaned microscope slide (25 x 75 mm) and covered with a siliconized coverslip. The slide, protected by two layers of bibulous paper, was pressed for about 10 seconds with a 2.5 Kg. copper rod which was held vertically in loosely fitting clamps. The slide was placed, coverslip down, on a block of dry ice for 4 to 5 minutes, and the coverslip was removed quickly with a razor blade. The slide was immediately plunged into absolute ethanol for a few seconds and air dried. The slides were stained in carbol fuchsin for 7 minutes and subsequently dipped into each of a series of graded ethanols and xylene for 5 to 10 seconds each. The preparations were finally mounted in DePeX (G.T. Gurr Ltd.,

London, England) with precleaned coverslips.

V. Growth and Titration of Sendai Virus

1. Source of Sendai Virus

Seed virus was obtained from Dr. F.P. Nagler, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, and also from Dr. H. Harris, University of Oxford, London, England. These were stored at -76°C.

2. Growth of Sendai Virus

The reconstituted seed virus was passaged at a dilution of 1:10,000 in 10-11 day-old fertilized hen eggs by allantoic route. Virus dilutions were made in sterile physiological saline. For inoculation into the allantoic cavity, 0.1 ml. of virus inoculum which gave about 1 to 10 HAU (Hemagglutination units, see below), was injected through a small hole punctured previously in the egg shell. The site of injection was determined by candling the eggs in the dark and marking the upper margins of the air sac in the area opposite the side of the embryo. After swabbing with 95% ethanol, a 1.5 inch 22 gauge needle on a 1 ml. syringe was directed through the shell and down to the allantoic cavity for about 0.5 inch. The hole of the inoculation site on the egg shell was sealed with hot wax and the eggs were incubated for 60-68 hours at 37°C in a humidified incubator. After incubation, they were placed at 4°C overnight or until fully chilled. The allantoic fluid was collected by cutting away the shell over the air sac,

removing part of the overlying membranes and aspirating with a sterile pipette. The pooled allantoic fluids were stored at 4°C for cell fusion of passage.

3. Titration of Sendai Virus by Hemagglutination

Serial two-fold dilutions of harvested virus were prepared in 0.5 ml. volumes of physiological saline in 85 mm. x 10 mm. titration tubes. To each tube was added 0.5 ml. of 0.5% (packed volume) suspension of washed chicken red blood cells (RBC). Tubes were shaken and stood in a perpendicular position at room temperature for two hours. In this procedure the presence or absence of hemagglutination was indicated by the distribution of chicken RBC as they settled to the bottom of the tubes. Agglutinated cells were seen more of less evenly distributed in a thin layer over the entire bottom of the tube. In the absence of agglutination, cells were seen only in the center of the bottom in the form of a compact "button". Partial agglitination was indicated by intermediate types of patterns. The hemagglutination (HA) titer was taken as the highest dilution of virus prior to addition of chicken RBC suspension, which produced complete agglutination of the RBC.

4. Ultraviolet Light (UV) Inactivation of Sendai Virus

Three ml. of virus in a 6 cm. diameter Petri dish was placed 15 cm. from a General Electric type G 8T5, UV tube and exposed for 10 minutes with agitation at every 2 minute intervals. The intensity of radiation incident on the surface of the fluid at this distance was given by the manufacturer as 0.14 watts per squre feet.

VI. Cell Fusion and Cell Separation

Single tumor cells were fused by a technique essentially as described by Harris et al (1966). Prior to cell fusion, dissociation of tumor cells was obtained by forcing the tumor fragments in and out of a syringe without a needle and finally pressing through a 23 guage needle. A small quantity of HBSS was used to facilitate this operation. Clumped cells were allowed to settle in a tube for 1 to 2 minutes and the supernatant, which was virtually a suspension of single cells, was removed to another tube. This step was essential to obtain cell preparation suitable for cell fusion. Cells, suspended in 10 to 15 ml. of HBSS, were centrifuged at 1000 rpm for 10 minutes in a clinical centrifuge and resuspended in HBSS. Aliquots of the cell suspension were removed and tested for cell viability by trypan blue dye exclusion. Viability was expressed as the percentage ratio of unstained cells to total cells counted. Cell suspensions with over 80% viability were used for fusion. One to 2 million cells were resuspended in 1 ml. UV inactivated Sendai virus containing approximately 2500 HAU. The mixture was allowed to stand in an ice bath with frequent agitation for three changes of virus suspension; each change remained in the cold for about 15 minutes. At the end of the period, the excess virus in the cell suspension was washed off with prewasrmed (37°C) HBSS containing 15% of fetal calf serum (Grand Island Biological Company, Grand Island, New York). The cells with about 4 ml. of medium remaining in the tube, were transferred to a water bath or an incubator at 37°C for 30 to 50 minutes

with occasional agitation. It was found that the concentraion of fused cells could be increased by the use of discontinuous Ficoll gradients. Ficoll solutions were prepared as follows, 100 ml. PBS without Ca^{++} and Mg^{++} in a 500 ml. beaker were prewarmed in a 56°-69° water bath. 100 gm. Ficoll was added to the solution and mixed by stirring until it turned to a "marsh-mallow-like" soup. It was left at room temperature overnight. The density of the stock solution and following densities of the solutions in gm/ml, 1.110, 1.0900, 1.0700, 1.670, 1.0660 1.0600, 1.0500, were prepared by a hydrometer at room temperature. PBS without Ca^{++} and Mg^{++} was used as diluents throughout.

Solutions were sterilized by pasteurization. Solutions were kept at 4°C, but always warmed to room temperature before use.

Each gradient solution in amount of 0.5-1 ml. was put into 10 ml. tubes used in SW 39 or SW 50 rotors, by holding the tip of the pipette against the side of the tube and allowing the solution to flow slowly. The most dense solution was first introduced into the tube, and others followed in high to low density taking care to avoid bubbles. The cell suspension (0.5-1 ml.) were carefully overlayered without mixing at the interphase. The tubes were centrifuged for 15 minutes at 2000 rpm. in Spinco Model L2.

After centrifugation, two major visible bands in the tube were observed: the upper band and the lower band which appeared much more diffuse and wider than the former one. The upper band contained mainly single mononucleate cells while the lower one was predominantly of multinucleate fused cells. Fractions were initially collected by puncturing the centrifuge tube with a Buchler drop collecting unit (Buchler Instruments, Fort Lee, N.J.). However, the rate of dropping was slowed down and eventually stopped as the band of the fused cells was approaching to the bottom. In addition, some trace of cells was dragged along the wall of tube when the lower band was descending, although the tube had been siliconized before use.

Because of these technical difficulties, the layers were removed from the lower band then the upper band, using a 4 inch canula fitted to a 2 ml. disposable syringe or using a pipette with fine end and neck. The bands were sufficiently far apart, but it was not possible to remove the band without contamination with cells of the adjacent band.

VII. Immunization and Challenge Prodecures

Each immunizing dose of fused cells was estimated on the basis of total number of nuclei instead of the number of cells. A million nuclei of fused cells was used for the dose of tumor immunization. The concentration of fused cells was determined by subtracting the number of single cells obtained before fusion. The inoculum of estimated dose was concentrated into 0.2 ml. for injection.

Active immunization of A/Jax male mice was accomplished by intramuscular injection of the prepared immunizing dose of one million nuclei. One to three injections were given into a hind leg each time, left and right alternatively, of each mouse at 10 day intervals.

Tumor challenge in the treated and untreated control mice were performed 10-12 days after the last immunization by intramuscular injection of 0.1 ml. tumor suspension containing 1×10^5 viable cells. Subsequent challenges were carried out at 15 day intervals.

In addition to immunizing mice with Sendai virus-fused cells, two other techniques were used for comparison. These involved the use of neuraminidase treated tumor cells or cells which had received γ-irradiation.

In the experiments using neuraminidase, the enzyme preparations were obtained from two sources. The first one was prepared from Clostridium perfringens ("NEUP", lot 7LA,

Worthington Biochemical Corp., Freehold, N.J.) in powdered form with activity of 0.5 units per milligram. Before use, it was made into suspension with 0.1 M sodium acetal-acetic acid buffer of pH 5.0. The second enzyme preparation was a purified filtrate from culture of Vibrio cholerae. It was supplied in an acetate buffer at pH 5.5 containing calcium ions and had a quoted activity of 500 units/ml. (Behrinwerke, Batch #966E, Hoechest Pharmaceutical Co., Kansas, Mo.)

After washing twice in HBSS, 10-15 x 10^6 MC tumor cells were incubated in 1.0 ml. of either acetate buffer, pH 5.5 in undiluted 500 units/ml. neuraminidase, or in neuraminidase previously heated to 60°C for 30 minutes. The tumor cells were incubated at 37°C for 45 minutes with periodic shaking and then washed again 3 times in HBSS. Cell viability was assessed by trypan blue dye exclusion before and after incubation. The cells then served as immunizing inocula. Each mouse received 1 x 10^6 cells intramuscularly. Procedures for immunizing and challenging were similar to those used when fused cells served as the immunizing dose.

In the experiments using gamma irradiated cells, the tumor suspension in HBSS was exposed to 15,000 rads in an open petri dish employing a 2000 Curie Cs ^{137} source. The dose of irradiation used was according to Revesz (1960) who suggested that immunization with tumor suspensions irradiated with 14,000 to 20,000 rads is very effective against tumor induced by chemical carcinogens.

VIII. Delayed Hypersensitivity Test

Tumor cells (or other materials to be tested) were injected intradermally into the hind foot pads of tumor immunized mice, tumor bearing mice, or normal control mice, using a tuberculin syringe and 27-gauge needle to deliver 0.02 ml. Specific MC tumor cell suspension or extract was injected into the left foot, and other materials (e.g., non-specific tumor, SP, or physiological saline) was injected into the right foot for direct comparison. The foot thickness was measured before injection, and 24 and 48 hours after injection, with a dial gauge ("Quickest" caliper, Type A.02A, H.C. Kroplin GmbH, Hessen, Germany). The foot-pad reactions of some representative mice were examined microscopically, after the foot was amputated and processessed with fixing, decalcifing, sectioning and staining in hematoxylin and eosin.

IX. Adoptive Transfer of Immunity

Spleens and regional lymph nodes were obtained aseptically from immunized mice. Each spleen was grouped separately and lymph nodes were pooled from 5 to 10 mice each time. The resulting suspensions were washed twice in HBSS and counted in hemocytometer (with 10% acetic acid in water). All suspensions from different groups demonstrated greater than 90% viability. Dissociated cells of MC tumor, spleen and lymph node were made according to that described earlier for the tumor cells. The protocol of three experiments will be described in Results.

X. Spleen Cell Culture and Tritiated Thymidine Incorporation Assay

1. Spleen Cell Culture Techniques

(1) Reagents

(a) Eagle's Minimum Essential Medium (MEM) Spinner (10X): Ten fold dilution of the medium (Grand Island Biological Company, Grand Island, New York) was prepared from the stock solution in sterile deionized distilled water.

(b) Normal Rabbit Serum (NRS): NRS was obtained from normal adult rabbits in a standard procedure, and stored at -20°C. The serum was heated to 56°C for 30 minutes and added to medium to a final concentration of 5%.

(c) Glutamine: L-glutamine (200 mM) solution (Grand Island Biological Company) was added to the medium to a final concentration of 1%.

(d) Sodium Bicarbonate Solution: This was prepared as a 7.5% solution of sodium bicarbonate in distilled water and was sterilized by millipore filtration. The solution was added to medium to a final concentration of 2%.

(e) Antibiotics: Two antibiotics, penicillin-G sodium and streptomycin sulfate (General Biochemical Company, Chargrin Falls, Ohio) were used. Stock solutions containing penicillin at 2×10^3 IU/ml., and streptomycin at 4×10^3 µg/ml. in deionized distilled water were prepared and sterilized by millipore filtration and stored at -20°C until use. Each antibiotic solution was added to culture medium at the concentration of 1 ml. per 100 ml. of medium.

(f) Phytohemagglutinin (PHA)-p: PHA-p (Difco Laboratories, Detroit, Michigan) was added to the culture medium at dilution of 1:25 and 1:250 from the stock to give an optimal and suboptimal stimulating dose, respectively, for mouse lymphocytes (Dent, 1969).

(2) Spleen Cell Culture

Cells were obtained by removing the spleens from mice under sterile conditions. The spleen was minced with a scissors and gently dispersed using a loosely fitting pestle in a Potter Elvehjgem homogenizer. The cell suspension was then drawn through a 25 gauge needle to produce a suspension of single cells. The lymphoid cells were counted and diluted with culture medium to a final concentration of approximately 1×10^6 cells per ml. Each tube contained 2 ml. of cell suspension. Triplicate tubes were made for each group.

2. Deoxyribonucleic Acid (DNA) Synthesis by Tritiated thymidine (^{3}HTdr) Incorporation

(1) Reagents

(a) Tritiated Thymidine: 3HTdr (New England Nuclear Corporation, Boston, Mass.) had a specificity of 6.7 c/mM in sterile deionized distilled water.

(b) Scintillation Fluid: The solution was prepared by adding 20 gm. of permablend^{TM} (Packard Instrument Company, Inc., Downer Grove, Illinois) to 4 liters of toluene.

(2) Scintillation Counting: The incorporation of ^{3}Htdr by the cells in culture was estimated as follows: The spleen

cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 43 hours. Two μc of tritiated DNA precursor was added to each 2 ml. culture in tubes at a volume of 0.2 ml. and incubated for another 5 hours. For termination, 0.15 ml. cold thymidine (3 mg/ml) and 5 ml. normal physiological saline were added to each culture tube. The preparations were then centrifuged and the supernatants discarded. The cell pellets were kept frozen at -20°C for at least 1 hour and digested twice with 5 ml. 10% cold trichloracetic acid (TCA) followed by one extraction with 5 ml. 80% cold ethanol. Finally, the deposited cells were dissolved in 0.5 ml. Nuclear Chicago Solubilizer (NCS) (Nuclear Chicago Corporation, Des Plaines, Illinois) and the solution was transferred to screwtop vials. The content of tritium was estimated in a Packard Model 3310 liquid scintillation spectrometer using 10 ml. of scintillation fluid. The amount of quenching was assessed for each sample by means of an external standard. The results were calculated as counts per minutes per culture.

XI. Titration of Anti-tumor Agglutinins

Blood from tumor immune mice and normal virgin mice of comparable age was collected separately from the retro-orbital sinus by means of a sterile Pasteur pipette previously heparinized. The blood was emptied into test tubes, allowed to clot, then centrifuged to obtain the serum. Serum was stored at -20°C.

Tumor cells were washed 3 to 4 times with a large volume of PBS, using low speed centrifugation (450 rpm) between washes, and counted with a hemecytometer. For tube agglutination, 0.25 ml. of tumor cell suspension containing 5×10^6 cells/ml. was added to an equal volume of each serial 2 fold dilution of serum. After being left undisturbed for 2 hours at room temperature, the tubes were gently tapped and the degree of agglutination was examined with a 7x magnifying lens. For slide agglutination, 2 drops of serum dilution and 2 drops of tumor cell suspension containing 5×10^7 cells/ml. were mixed within a circle marked with a wax pencil on a microscope slide. The slide was gently rocked for 15 seconds, then left undisturbed for 3 minutes and was rocked again briefly; a striking clumping would appear in positive tests, whereas in a negative test the cells would form a uniform suspension. All cell and serum dilutions were made in PBS.

XII. Indirect Immunofluorescent Antibody Technique

The indirect fluorescent antibody test was performed on tumor frozen sections essentially described by Möller (1961) for the demonstration of mouse isoantigens. Tumor or liver tissues were removed from the animals, and washed in PBS. The frozen sections of 5-6 μ in thickness were made in Tissue-Tek Microtome-Crystat (Ames Company, Elkart, Indiana) and mounted on the pre-albumin coated slides. 0.2 ml. of mouse antiserum or normal serum was flooded evenly on the specimen of the slide and incubated at 37°C for 20 minutes

in humidified atmosphere. The slides were washed three times in PBS. Each slide was then flooded with 0.2 ml. of undiluted fluorescein isothiocyanate conjugated mouse 7S gamma-globulin (Pentax Inc., Montreal, P.Q.). After being washed twice in PBS, the slides were coverslipped with one or two drops of the solution consisted of glycerine and isotonic phosphate saline (pH 3.3), 1:1 (V/V). The slides were stored at 0°C until examined with a Nikon's fluorescence microscope under dark ground illumination.

XIII. Effect of Antiserum on Tumor Growth

The effect of serum from immunized mice was tested by incubating MC tumor cells with the test serum for 40 minutes at room temperature before injection. Undiluted and 1:5 dilution sera were used. A million cells were incubated with 1 ml. pooled immune serum. Serum from normal mice of comparable age was used for the control experiment. After incubation with serum, a hundred thousand cells were washed once in PBS and then injected into each animal.

RESULTS

I. Features of Tumors

1. Gross Appearance of MC Tumor

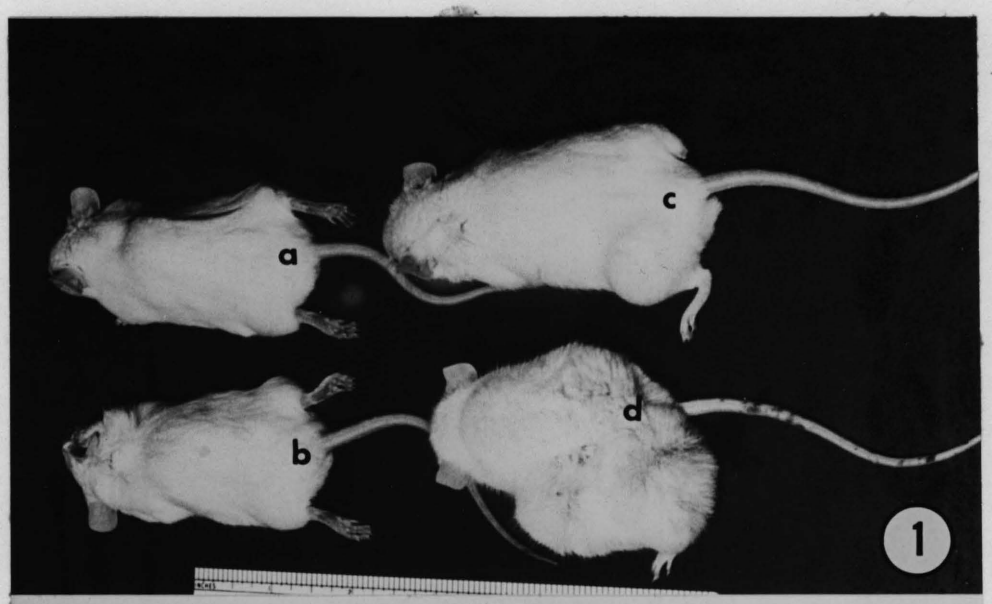
MC tumor was experimentally transplanted to the lateral side of the hind legs of A/Jax mouse by intramuscular injection. Fig. 1 illustrates the mice at different stages of tumor development following injection of approximately 1 x 10^6 cells. The tumor began as a small nodule, which was palpable as the animals started limping (Fig. 1.b) and reached maximal sizes of 4 to 5 cm. in diameter with irregular shapes (Fig. 1. d). As the tumor developed ulceration of epidermis often occurred and hemorrhage and necrosis followed. These tumors sometimes became so large that they protruded from the thighs resulting in degeneration of the lower part of the affected legs. When ulceration of the epidermis did occur, the tumor usually had a doughnut-like appearance with a brownish necrotic center (Fig. 2).

2. Light Microscopic Examination

Light microscopic observations of the hematoxylin and eosin stained MC tumors showed that the tumor was an undifferentiated cellular fibrosarcoma. The sarcomas exhibited a mass of packed cells arranged in whorled array or bundles running in different directions (Fig. 3 and 4). The tumor was composed of round, oval, polyhedral or spindle-shaped cells

Fig. 1. Tumor growth on the left legs of A/Jax male mice after MC tumor transplantation with approximately 1×10^6 cells. a. Control untreated mouse. b. Mouse at 6 days after tumor injection. Tumor growth is not visually noticeable; it could be palpable by hands. c. Mouse at 11 days after tumor injection. Growth of tumor is noted on the left leg. d. Mouse at 27 days after tumor transplantation. Outgrowth of a MC tumor and its irregular shape are evident on the left leg expanding to left lateral side of the body.

Fig. 2. Exposed MC tumor on the left leg of a mouse at 15 days after tumor injection at a dose of 2×10^6 cells. Necrotic part (arrow) of the tumor is noted.


a
b
c
d
1

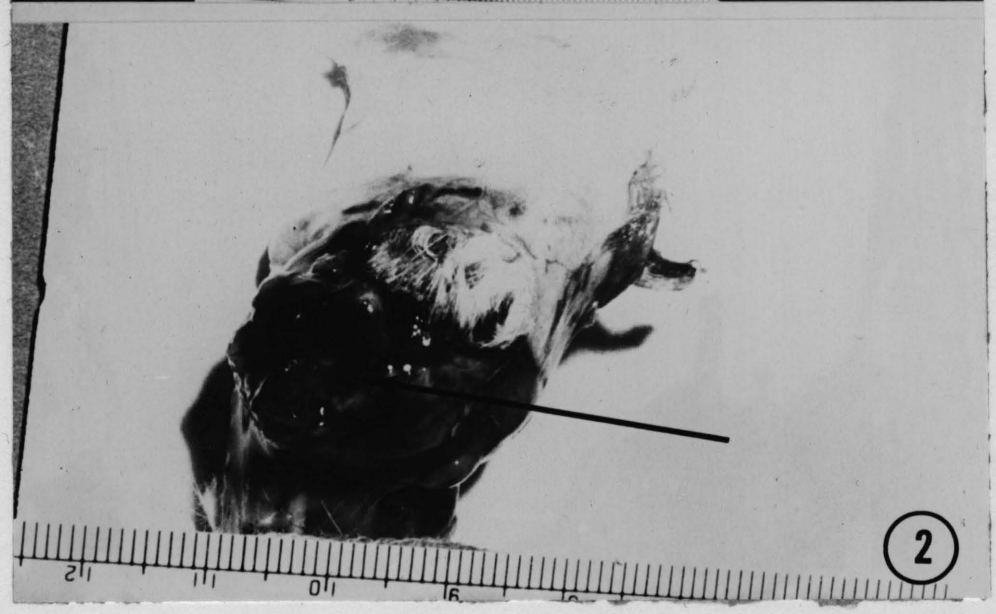

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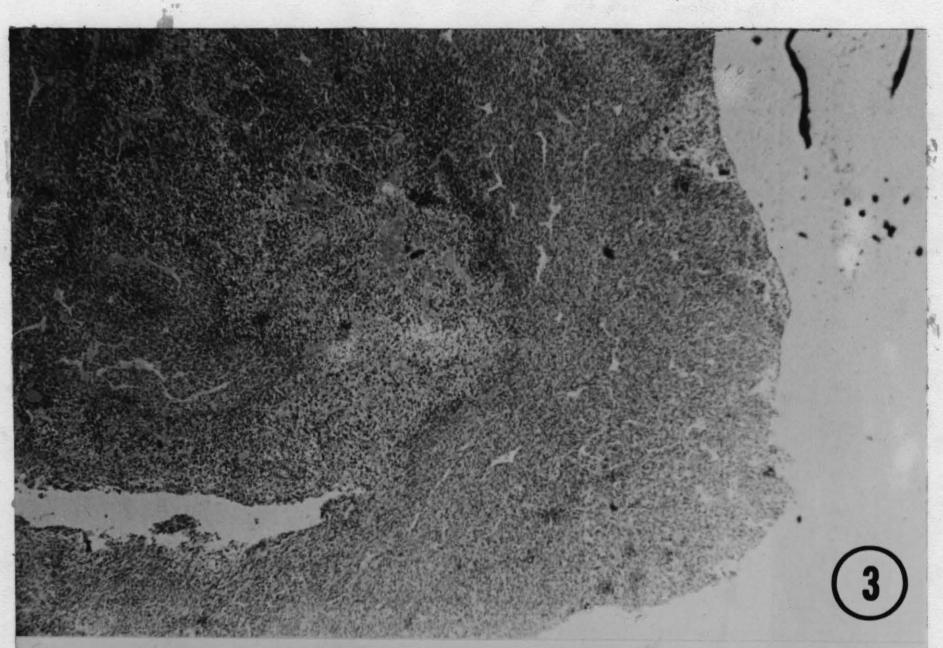
Fig. 3. Photomicrograph of MC tumor. Hematoxylin and eosin stain. x 40. Fibrosarcoma. There are several areas of necrosis in the central part.

Fig. 4. Photomicrograph of MC tumor. Hematoxylin and eosin stain. x 150. This tumor is composed of spindle, round, oval and polyhedral shapes of cells arranged in sheets, interlacing bundles and whorls.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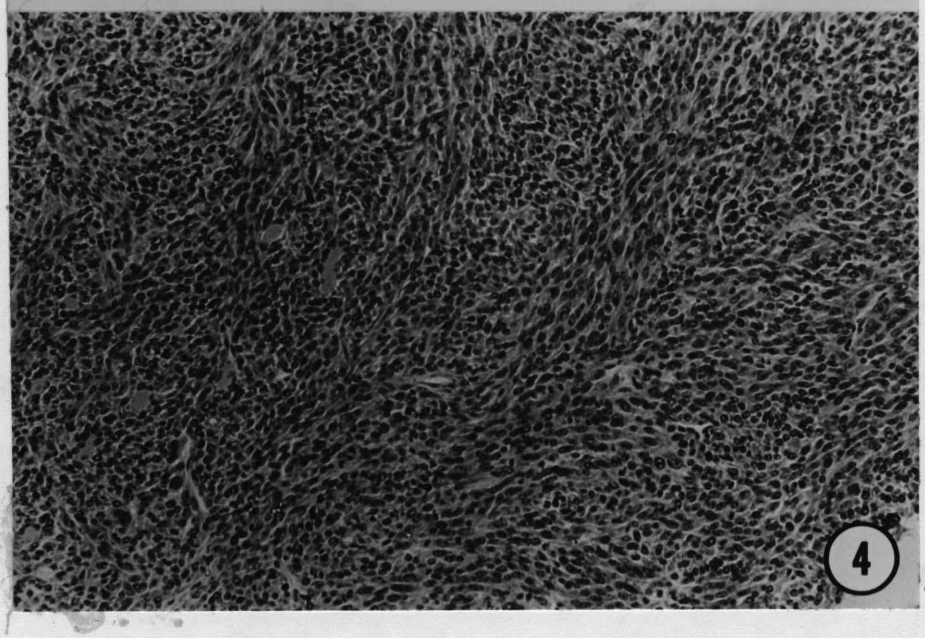
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varying in diameter from 10 to 13 microns. The nuclei appeared pleomorphic, with visible nucleoli. The nuclear/cytoplasmic ratio varied from cell to cell. Mitotic figures were frequently seen; however, the incidence of mitosis varied from one area to the other. All of the tumors examined had a little collagenous stroma and were relatively avascular. Inflammatory cells were noted only in area of necrosis (Fig. 3). In histological sections, no definite encapsulation of MC sarcoma was observed. Some degree of invasiveness to the surrounding muscular fibers was noted. Sections of spleen, lungs, liver, lymph nodes, thymus and kidney from the tumor bearing mice at various stages were made and examined microscopically. No detectable metastases were found in these organs.

3. Electron Microscopic Examination

The ultrastructures of the MC tumor were examined in several passages in the course of the investigation. Cells were of elongated shapes, their cell periphery being irregular with few microvilli-like projections (Figs. 5 and 6). The mode of contact between tumor cells varied resulting in intercellular spaces of varying size. The tumor was made up of densely packed cells with little intervening extracellular materials. Collagen fibrils were infrequently seen (Fig. 7). The overall appearance of the tumors suggested that they were composed of one general cell type. The nuclei of tumor cells were irregular in outline. In addition to distribution of chromatin along the nuclear

Fig. 5. Electron micrograph of MC tumor cells. Portions of 4 cells. Normal appearance of cell structures. nm, nuclear membrane; chr, chromatin; v, vesicles; ER, endoplasmic reticulum. The nuclei (N) are heterogeneous showing areas of dense and diffuse chromatin. X36,000.

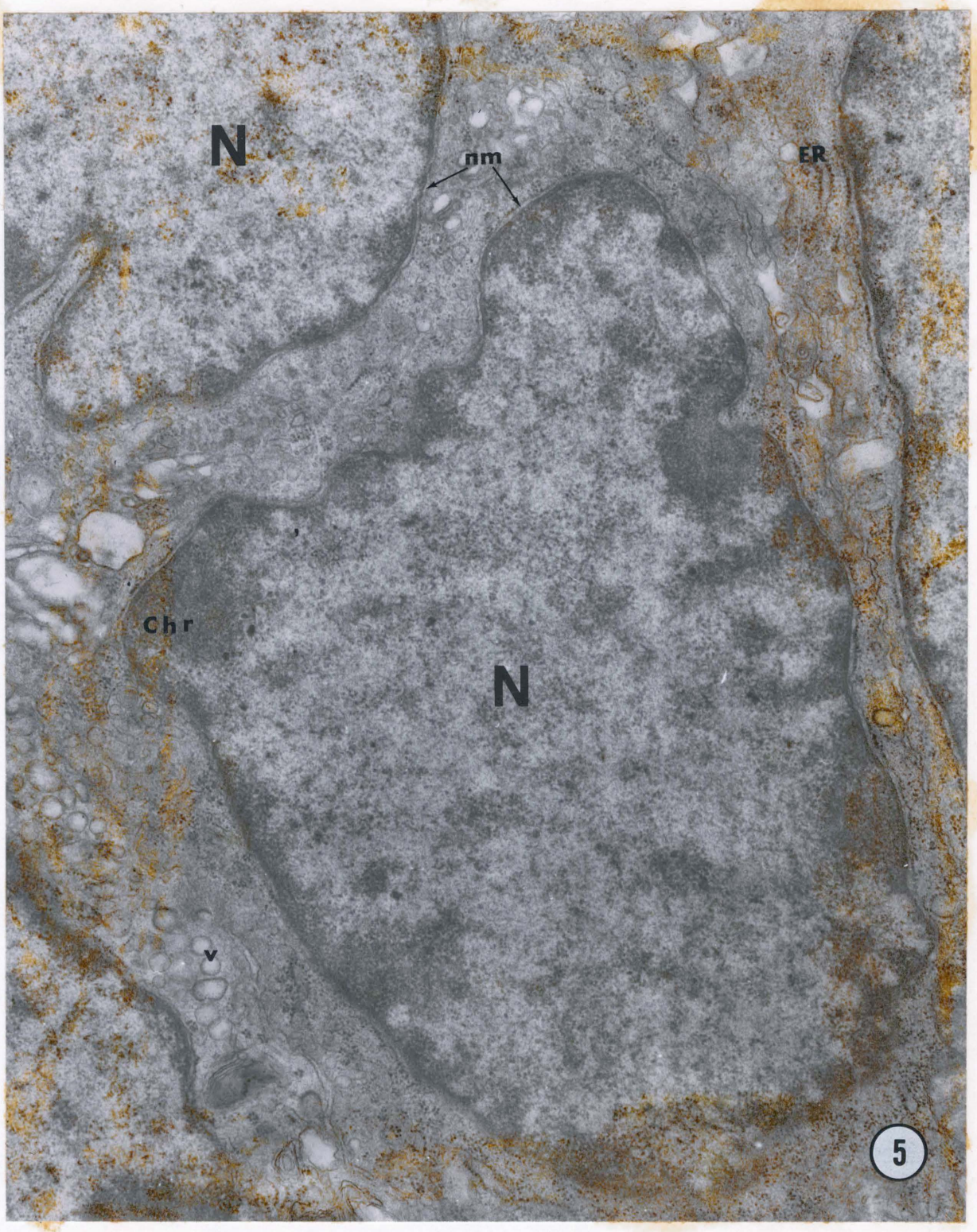
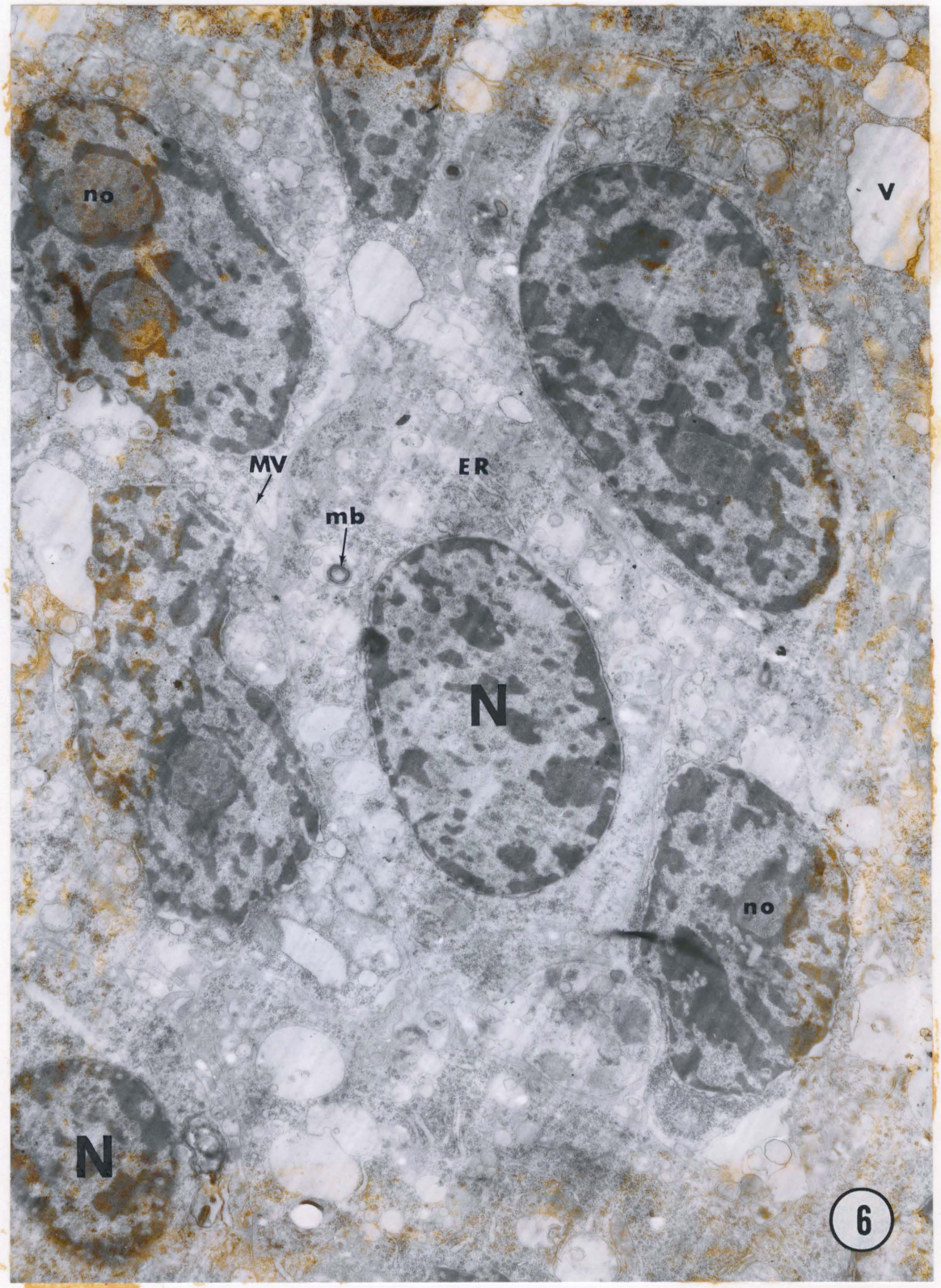

N
nm
ER
Chr
N
v
5

Fig. 6. Electron micrograph of MC tumor showing an area containing degenerating cells.
V, Vacuole; no, nucleolus; mb, membranous body; MV, microvilli-like projection; ER, endoplasmic reticulum.
The nuclei (N) are heterogeneous showing areas of dense chromatin. X7,500.


no
V
MV
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mb
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6

membrane, there was some chromatin irregularly distributed in clusters in the nucleus (Figs. 5 and 6). The size and localization of nucleoli within the nuclei varied considerably.

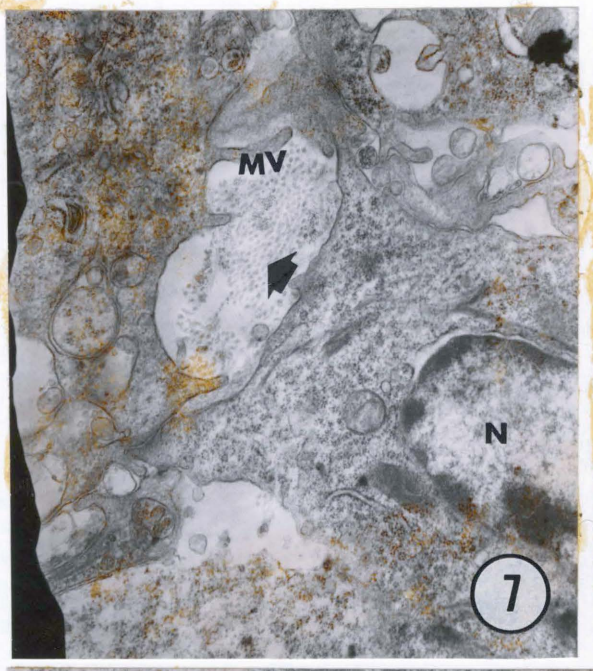
Cytoplasm of most tumor cells was characterized by paucity of rough endoplasmic reticulum. Golgi apparatus was noted in the tumor population. Mitochondria were found in various sizes and shapes. However, mitochondria were rarely seen in degenerating tumor cells(Figs.6&8),which were characterized by the presence of many various sizes of vesicles, vacuoles and some membranous bound bodies. Membraneus bound bodies (Figs. 6 and 9) were structually consistent with those identified as microsomes.

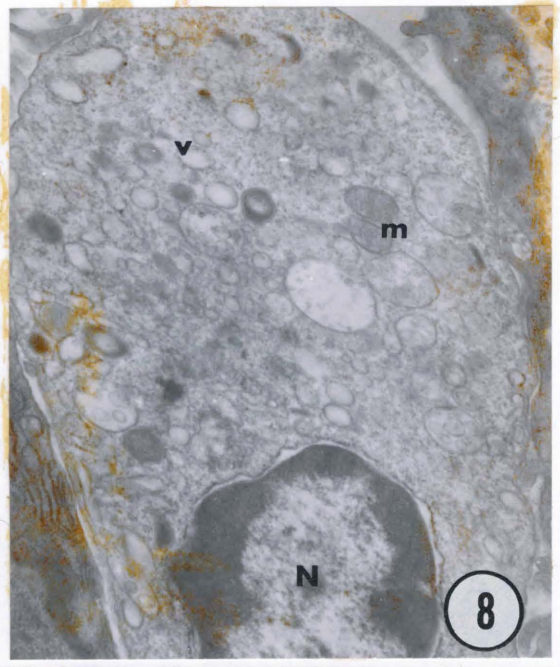
Figs. 7, 8 & 9. Electron micrographs of MC tumor cells.

Fig. 7. Portions of two or more neighbouring cells with associated collagen fibers (large arrow). Microvilli-like projections, MV, extending from the cell periphery (small arrow) are noted. N, nucleus. x15,000.

Fig. 8. Portion of a MC tumor with vacuoles and vesicles scattered in cytoplasm. N, nucleus; v, vesicles; m, mitochondria. x13,000.

Fig. 9. Portion of a MC tumor cell showing various cytoplasmic structures. db, dense body; ER, endoplasmic reticulum; mvb, multivesicle body; m, mitochondria; nm, nuclear membrane, Pn, perinuclear materials; sm, swellon mitochondria with no appearance of cristae; V, vacuole; v, vesicle. x26,000.


MV
N
7


v
m
N
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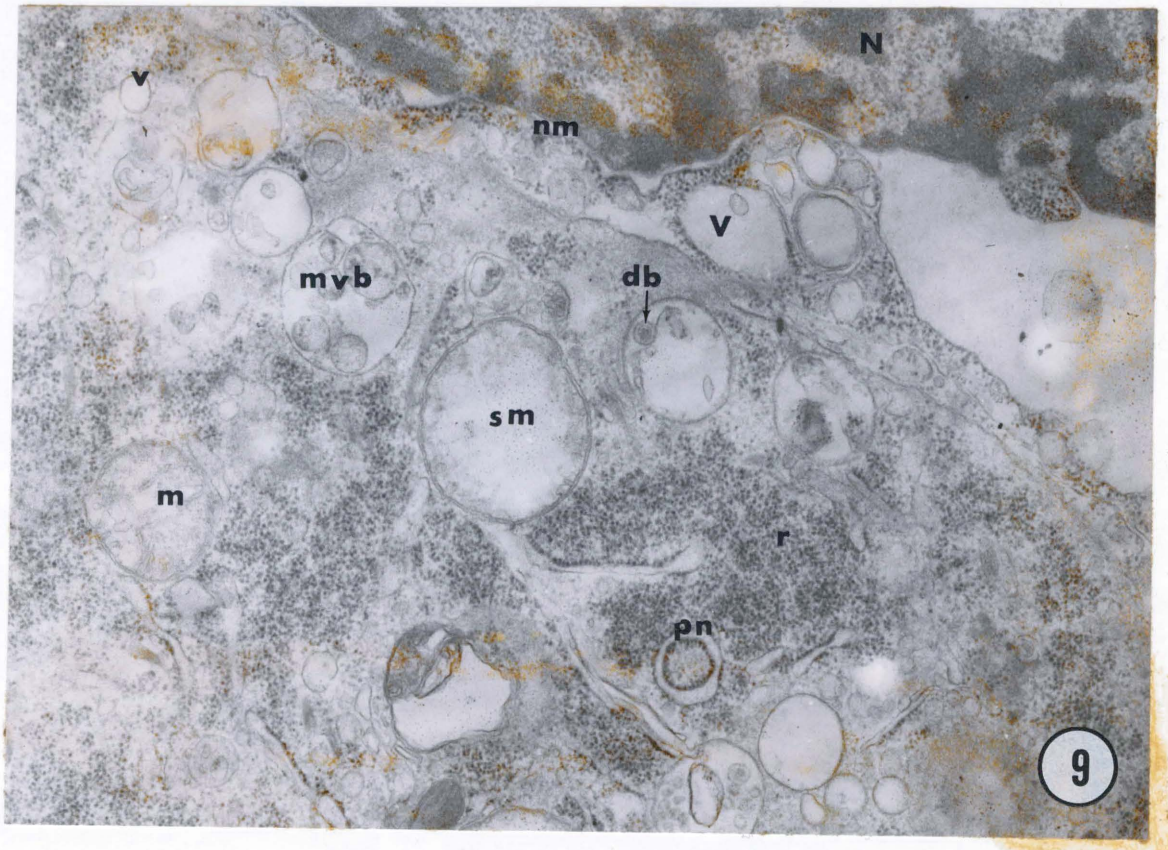
N
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db
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9

4. Chromosomal Analysis

Diffusion chambers were used to grow the MC tumor cells or mouse embryonic cells in mouse peritoneal cavity. The cells were treated with colchicine in vivo and harvested for chromosomal analysis. The tumors at three different passages were studied with respect to their karyological features. The three passages were designated as P_{x+5}, P_{x+19}, and P_{x+29}, where x represents unknown passage number of tumor transplantation in vivo since 1961, and the numbers, 5, 19, and 29 indicate the passage numbers since the author strated working on this tumor. The MC tumor cells were characterized by stemline karyotypes of 43 chromosomes, including always one or infrequently two large metacentric marker chromosomes. Fig. 10 shows a karyotype and the metaphase plate of a cell from embryonic tissue. The cell contains 40 chromosomes of normal shapes without any observable marker chromosome. All metaphase elements appear to be telocentric. Examples of idiograms and metaphase plates of three MC tumor cells are shown in Figs. 11 to 13. Fig. 12 shows a pseudo-diploid cell with 40 chromosomes and Fig. 13 a hyperdiploid cell with 43 chromosomes. Both of the cells include one marker chromosome of a similar type. Fig. 13 shows a near-tetraploid cell containing 86 chromosomes including 2 metacentric markers.

The frequency of polyploid tumor cells including near 4n, near 8n, and over 8n (where n stands for haploid number; in mouse, n=20) in P_{x+5}, P_{x+19}, and P_{x+29} is 13.16%, whereas the frequency of near-diploid cells is 86.84% (Table I). Among

Fig. 10. Metaphase spread and karyotype of a cell from diffusion chamber in vivo culture of embryonic cells of A/Jax mouse. Carbol fuchsin stain. The cell contains 40 telocentric chromosomes.

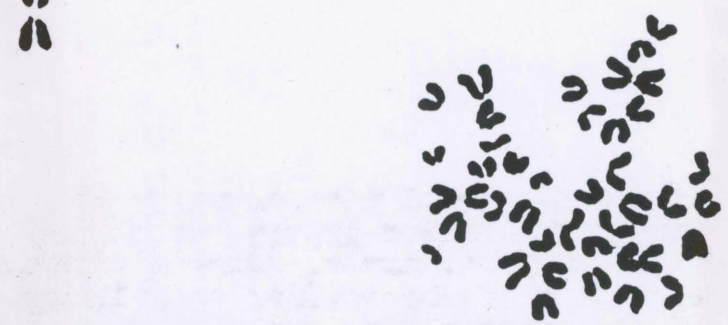
Fig. 11. Metaphase spread and karyotype of a cell from diffusion chamber in vivo culture of MC tumor cells of A/Jax mouse. Carbol fuchsin stain. There are 43 chromosomes including one metacentric marker chromosome.

(10)

(11)

Fig. 12. Metaphase spread and karyotype of a cell from diffusion chamber in vivo culture of MC tumor cells of A/Jax mouse. Carbol fuchsin stain. There are 40 chromosomes including one metacentric marker chromosome.

Fig. 13. Metaphase spread and karyotype of a cell from diffusion chamber in vivo culture of MC tumor cells of A/Jax mouse. Carbol fuchsin stain. There are 86 chromosomes including two morphologically similar metacentric marker chromosomes.



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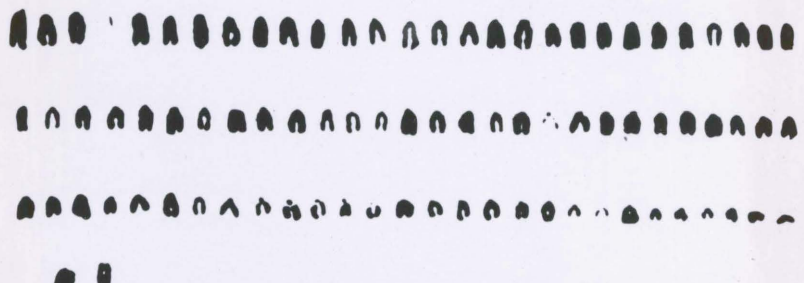

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Table I. Frequency of various ploid cells in MC tumor

Transplant Passage number in vivo	% of cells	% of polyploid cells				Total number of cells observed
	Near 2n	Near 4n	Near 8n	> 8n	Total	
P_{x+5}	90.31 (289)[a]	9.06 (29)	0.63 (2)	0.00 (0)	9.69 (31)	320
P_{x+19}	82.58 (109)	15.15 (20)	2.27 (3)	0.00 (0)	17.42 (23)	132
P_{x+29}	84.21 (176)	12.44 (26)	2.87 (6)	0.48 (1)	15.79 (33)	209
Total number of cells	574	75	11	1	87	661
%	86.84	11.35	1.66	0.15	13.16	100

a. Figure in parenthesis represents number of cells.

the former, the frequency of near-tetraploid cells was highest (11.35%) and those of near 8n and over 8n, the second (1.66%) and the third (0.15%), respectively. As can be seen in Table I, overall chromosomal constitutions in the tumor cells from P_{x+5} to P_{x+29} did not change considerablt, though a slight tendency of increase towards near-tetraploidy through the serial passages is noted. The results of chromosomal analysis on the A/Jax mouse embryonic cells, representing normal mouse cells, with the same technique is shown in Table II.

5. Tumor Dosage Experiment for Progressive Growth of MC Tumor

Since large numbers of tumor cells may overwhelm the host's response and too few cells may not grow at all, experiments were carried out to find out an appropriate dosage of MC sarcoma cells which would show visible growth in a moderate length of time. To find this range, the threshold dose for progressive growth of MC tumor was determined. Male A/Jax mice were given intramuscular injections of from 40 to 10 millions tumor cells in the lateral side of left hind leg. The animals were observed for three months. The results of the experiment are expressed in Table III in terms of (i) numbers of animals with tumor take/numbers of animals injected with tumor, and (ii) duration from tumor injection to the appearance of palpable tumor (days).

Table II. Chromosome count distribution of embryonic cells of A/Jax mice cultured in diffusion chambers *in vivo*

Chromosome No.	38	39	40	41	80	Total
No. of cells	1	2	66	0	3	72

Table III. Dosage experiment for growth of MC sarcoma in male A/Jax mice

Dosage	No. of mice developed tumor / No. of mice injected	
10×10^6	1/1	(4)***
5×10^6	1/1	(5)
1×10^6	1/1	(6)
6×10^5	2/2	(7, 7)
4×10^5	2/2	(7, 8)
2×10^5	1/2*	(8)
1×10^5	2/2	(9, 9)
8×10^4	2/2	(8, 9)
6×10^4	2/2	(8, 10)
4×10^4	2/2	(9, 10)
2×10^4	2/2	(9, 9)
1×10^4	2/2	(10, 10)
8×10^3	2/2	(11, 12)
6×10^3	2/2	(11, 13)
4×10^3	2/2	(12, 13)
2×10^3	2/2	(13, 13)
1×10^3	2/2	(14, 15)
8×10^2	2/2	(13, 15)
6×10^2	2/2	(15, 16)
4×10^2	1/2**	(16)
2×10^2	0/2	
1×10^2	0/2	
8×10	0/2	
6×10	0/2	
4×10	0/2	

* One of the mice died due to an unknown cause before the palpable tumor developed.

** Threshold

***Figure in parenthesis represents duration (in days) from tumor injection to appearance of palpable tumor.

Several conclusions may be derived from this experiment. When the palpable tumors were detected in the animals, the tumor shows a progressive increase in size until death of the hosts. Regression never occurred in a tumor which had once become palpable in the treated mice. Generally, as a large number of cells injected a shorter time was required for appearance of the tumors. Threshold for progressive growth of MC tumor was foumd to be 400 cells. It usually took about 6 to 7 days for a tumor to become palpable in mice after transplantation at the dose of 1×10^6 cells.

6. Specificity of MC Tumor with regard to Host Transplantability

MC tumor cells, ranged from 2×10^6 to 6×10^6 cells, were separatly injected into 5 to 15 of the following strains of mice, C3H f/HeHa, Swiss/HA/ICR, dd/s sach, BDF and C57B1. The treated animals were observed for two months. Tumor failed to take in all these animals tested. Although a temporary growth of the tumor was observed in some of the C3H f/HeHa mice, tumor regressed completely within about 3 weeks.

In view of the results obtained from the previous dosage experiment and the present tests, it seemsthat the MC sarcoma exhibited a strong strain-specific behavior with regard to its transplantability.

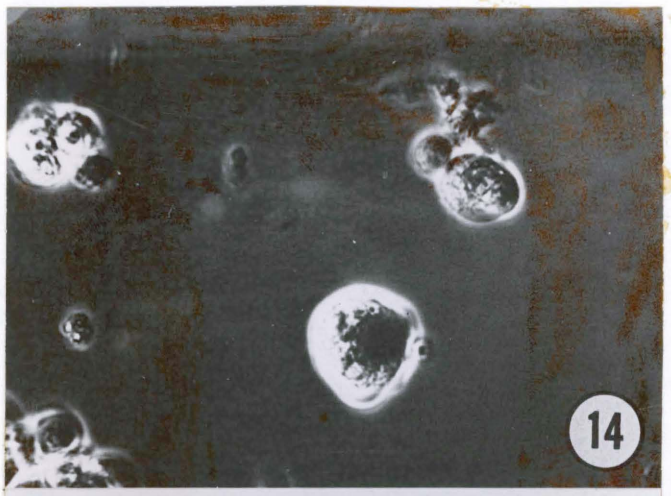
II. Active Tumor Immunization

1. Preliminary Experiment

Single MC tumor cells were treated with UV inactivated Sendai virus in cold and then incubated at 37° in HBSS, containing 15% fetal calf serum, with constant agitation. The cells in tubes were examined under an inverted microscope to check the rate of cell fusion. Cell agglutination was a precondition for cell fusion. The fused cells appeared to be relatively large, irregular in shape in an early stage and gradually become more spherical at the time of complete formation (Figs. 14 and 15). The treated tumor cell suspension consisted of single mononucleate cells, binucleate and other higher multinucleate cells. Ficoll discontinuous gradients were used to separate multinucleate cells from single mononuclear cells. Single cells were found to be contaminated in the collected "fused cells" in order of 5-20%. Scored from unseparated preparations the multinucleate cells were predominantly binucleate (53.8%) with higher nuclear counts being less frequent (Table IV). Polynucleate cells could be scored with as many as 50 nuclei. Figs. 16 and 17 illustrate dispersed single cells and multinucleate cells after cell separation, respectively. The single and various multinucleate cells with a higher magnification (x500) are shown in Figs. 18 to 25. As the cell preparation was treated briefly with hypotonic solution (distilled water) before fixation, cytoplasmic profiles of these cells could not be observed. Close associations among the nuclei in each fused cell were noted. Very frequently two or more neighbouring nuclei

Fig. 14. Photomicrograph of unstained MC tumor cells after treatment with UV inactivated Sendai virus. Note two fused cells at the upper left and lower middle areas. Several nuclei in each fused cell are vaguely visible. x450.

Fig. 15. Photomicrograph of unstained MC tumor cells after treatment with UV inactivated Sendai virus. Note the difference between the aggregation of several single cells (upper) and the fused cells (lower). Some of the cells in the aggregate might be in process of fusion. x450.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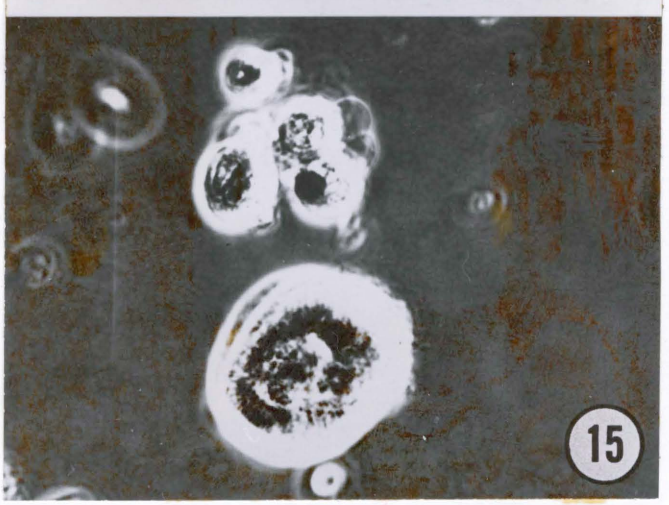
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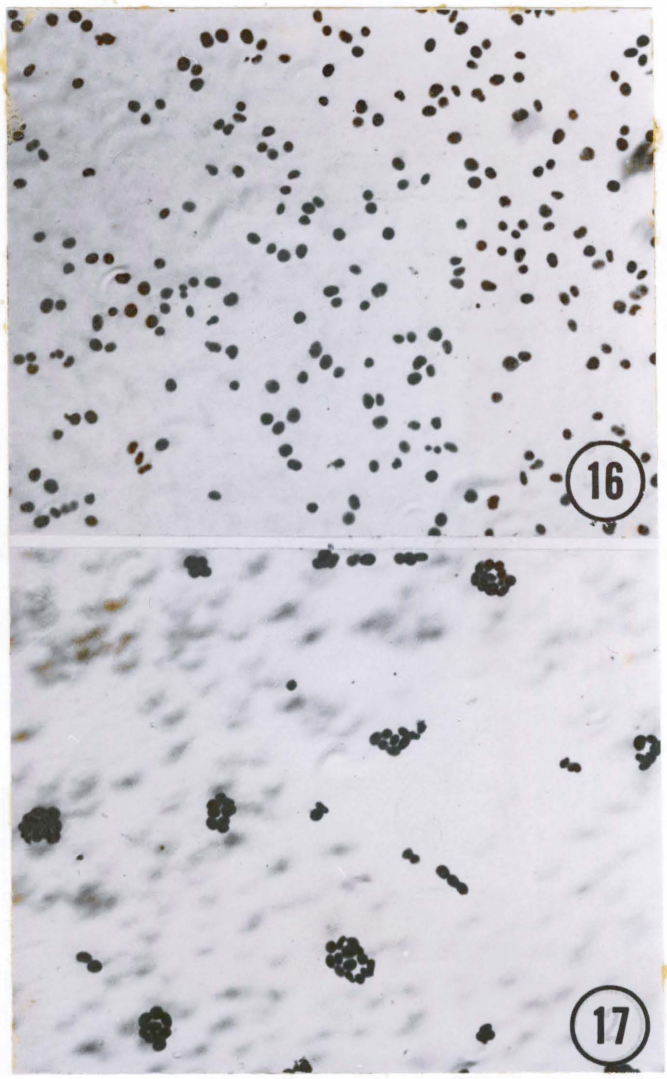
Table IV. Frequency distribution of multinucleate MC tumor cells following treatment with UV-irradiated Sendai virus

	Number of nuclei in fused cells																		
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	≥20
%	53.8	24.6	10.9	4.1	2.6	1.0	1.3	0.7	0.8	0.2	0.3	0.1	0.1	0.1		0.2	0.1		0.4

Fig. 16. Photomicrograph of stained preparation of single mononucleate cells collected after cell separation. Carbol fuchsin stain. x150.

Fig. 17. Photomicrograph of stained preparation of "fused cells" collected after cell separation. Carbol fuchsin stain. x150.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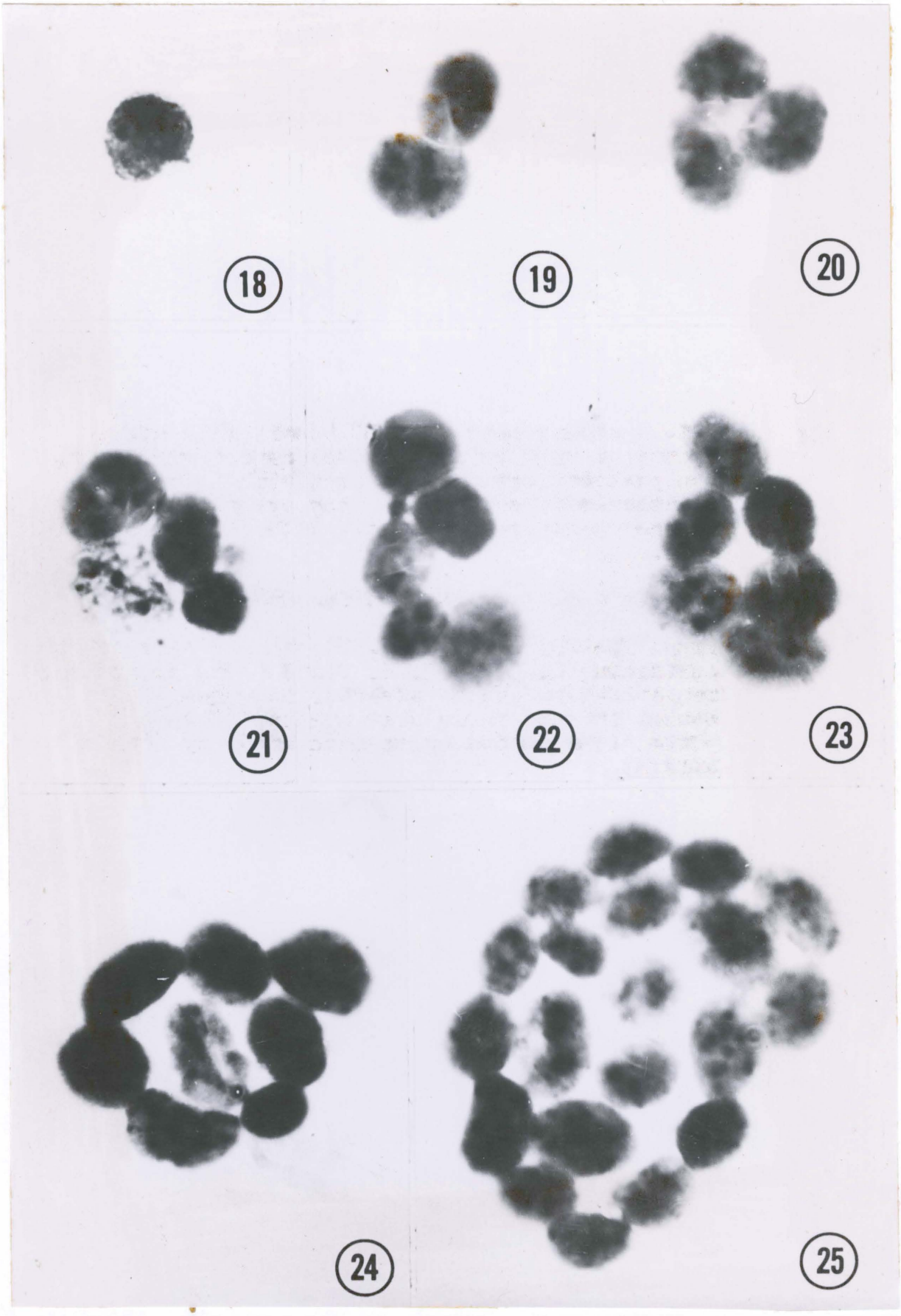
Figs. 18-25. Homokaryocytes of MC tumor cells after treatment with UV inactivated Sendai virus. As the preparations were treated with hypotonic solution before fixation, the cytoplasms of the cells are not seen. Carbol fuchsin stain. x500.

Fig. 18 shows a cell with one nucleus.

Figs. 19, 20, 21, 22, 23, 24, and 25 show cells containing 2, 3, 4, 5, 6, 8 and about 21 nuclei, respectively. Close associations among the nuclei in each fused cell are noted; some are obviously connected with each other by nuclear bridges.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were observed to be connected by nuclear bridges.

Six A/Jax mice were injected with such multinucleate cell preparation at a dose of approximately 1 x 10^6 nuclei into the thigh muscles of each animal. There were no signs of tumor development at the site of injection over a period of 15 days. However, 2 control mice which had been given 1 x 10^6 viable, non virus-treated single tumor cells from the same source used for cell fusion resulted in tumor development as usual. Fused cells, therefore, appreared to have lost their malignancy, though there was no loss of fused cell viability judging by trypan blue dye exclusion. It was thought that exposure to these fused, yet viable cells might produce immunity to the TSTA's of MC tumor. To test this proposition of host's immunogenicity, the mice were challenged with 1 x 10^5 tumor cells ($\simeq$1,000 LD_{50}'s) in the right leg 15 days after the fused cells were inoculated. Four out of 6 mice were protected from tumor development at the first tumor challenge. Fifteen days after this challenge, a second challenge was given to the remaining 4, and 2 developed tumors at the site of injection. Challenges were subsequently made of the two mice which remained tumor free, and the immunity to MC tumor was found to persist over 25 challenges until the time they were sacrificed for the experiment of adoptive transfer studies. For each challenge, two untreated mice served as controls. They were also injected with 1 x 10^5 viable tumor cells at each time, and 100% tumor takes were obtained in these control mice. On the basis of these observations, it became

clear that some degree of immunity against the immunizing tumor was induced in the treated animals.

2. Immunization Experiments

(1) Hyperimmunization with Three Repeated Immunizing Doses

A decreasing protective effect after the first and second tumor challenges in the animals from the preceeding pilot experiment might be due to a weak immune response after a single immunization. To build up a heightened tumor immune status in the host, three immunizing doses of fused cells were used instead of one. Twelve separate groups of experiments were performed. Each group consisted of 5, 6, or 10 mice. The animals were received three immunizing doses of fused cells at 10 days intervals. Fifteen days after the last immunizing dose was given, each animal was challenged with 1×10^5 viable tumor cells. For challenges, two control mice were always inoculated at the same time with the same dose of the tumor cells. The results of these experiments are summarized in Table V. A considerable degree of enhanced resistance in the treated mice was indicated by the outcome of the first challenge. Eighty-six per cent (92/107) of the mice failed to develop tumors. The resistant animals were subsequently given a second and third challenge of 1×10^5 live tumor cells at monthly intervals and the percentage of resistant animals was 77.6 (83/107) and 71.9 (77/107), respectively. All the control mice had 100% tumor takes.

Table V. Transplantation resistance to MC sarcoma cells induced in adult A/Jax mice by virus fused tumor cells

Experiment no.	# of animals tested	# of animals not developing tumor		
		1st challenge	2nd challenge	3rd challenge
1	5	5	4	4
2	10	9	9	9
3	10	7	6	6
4	6	5	4	3
5	10	8	8	8
6	10	8	7	7
7	10	9	9	9
8	10	10	10	6
9	10	9	7	7
10	6	6	5	5
11	10	8	6	6
12	10	8	8	7
	107	92(86.0%)	83(77.6%)	77(71.9%)

(2) Comparison of the Strengthof Tumor Immunogenicity with Those by Other Immunizing Techniques

Two other immunizing techniques, namely, injections of γ-irradiated tumor cells and neuraminidase-treated tumor cells in A/Jax mice were used to compare the degree of immunogenicity with that resulting from treatment with virus-fused tumor cells.

A pilot study was carried out with γ-irradiated tumor cells at an immunizing dose of 10^6 cells on a single occasion. All 10 animals used in this experiment developed tumor following challenge. However, a delay of 3 to 4 days in appearance of tumor was noted in these animals when compared with the controls. All animals used in the later experiments with γ-irradiated tumor cells were given three separate immunizing doses, as scheduled with fused-cell-immunization.

Two kinds of neuraminidase obtained from different sources were used in the experiments and found to give different results. Tumor cells which had been treated with neuraminidase prepared from Clostridium perfringens type V failed to induce observable immunity in 37 animals (in 3 separate experiments) to subsequent tumor challenges. The viability of tumor cells after treatment with this enzyme was foumd to be greatly diminished, as tested with trypan blue dye exclusion. The cytotoxicity could be attributed to impurity of the enzyme product containing some proteolytic enzyme.

Purified neuraminidase prepared from Vibrio cholerae culture was then used for subsequent experiments, as this kind

of preparation had been employed to demonstrate immune responses in animals against various types of tumors (Currie and Bagshawe, 1967, 1968, and 1969). There was no apparent effect on cell viability after the tumor cells were incubated with neuraminidase from this source.

The experiments using γ-irradiated-tumor cells and neuraminidase-treated tumor cells as immunizing doses were performed on three occasions under parallel conditions. At the same time, experiments 7, 8, and 9 using Sendai virus-fused cells were in progress (Table V). The results of all these experiments are summarized in (Table VI). These indicate that the protective effects of fused cells, γ-irradiated cells and neuraminidase treated cells upon first tumor challenge were 93.3%, 66.6%, and 56.7%; upon the second challenge, 86.6%, 36.6% and 46.6%; and upon the third challenge, 73.3%, 20.0%, and 36.6% respectively.

Two control mice were injected with 10^5 viable tumor cells in each challenge experiment at the same time and under the same conditions as the experimental animals. All these control animals developed tumors at the sites of injection.

(3) Survival of Treated Mice Which Failed to Reject First Tumor Challenge

Fifteen out of 107 treated mice in the twelve experiments of active immunization by fused cells failed to evoke a level of resistance such that the first tumor challenge was rejected (See Table V). The death of the pooled 15 mice and 14 control animals was recorded in Table VII. It is

Table VI. Comparative study on transplantation resistance to MC tumor cells induced in A/Jax mice by virus fused-, γ-irradiated-, and neuraminidase treated- tumor cells

Immunogen	No. of animals not developing tumor/Total animal tested (%)		
	1st challenge	2nd challenge	3rd challenge
Virus fused cells	93.3	86.6	73.3
γ-irradiated cells[a]	66.6	36.6	20.0
Neuraminidase treated cells[b]	56.7	46.6	36.6

[a] In the experiments using γ-irradiated cells, the tumor cell suspension in HBSS was exposed to 15,000 rads in an open petri dish empoying a 2000 Curie Cs^{137} source.

[b] $10\text{-}15 \times 10^6$ tumor cells were incubated in 1.0 ml. of acetate buffer, pH 5.5 in undiluted 500 units/ml. neuraminidase for 45 minutes at 37°C, and washed 3 times in HBSS.

Three separate immunizing doses were given to animals; 1×10^6 tumor cells, either γ-irradiated treated or neuraminidase treated, were used for each dose.

Table VII. Survival of fused cell treated and normal mice after tumor challenge (days)*

Mice	Number	1-26	27-30	31-34	35-40	41-50
Treated	15	1	2	1	8	3
Normal	14	0	12	2	0	0

*The results are based on the pooled data from the immunization experiments, 2. (1), in which the fused cell treated mice, 15 out of 107, failed to become resistant to the first tumor challenge. Therefore, the mice, both treated and control, listed in this Table died of tumor.

apparent that control mice died at 29 days, whereas immunized mice receiving the same challenge dose of tumor cells had a definitely prolonged longevity (mean survival; 37 days). The small but definite degree of protection could be appreciated from these data. It was noted that there is individual variation in the response from mouse to mouse, especially in the treated group.

(4) Persistence of Tumor Immune Status

Persistence of the immune state against MC tumor in the immunized animals with the three methods was examined by the outcome of repeated tumor challenges at 15 days intervals for the first 5 challenges, and monthly in the later stages up to 18 challenges. The immunoprotective effects of the immunizing treatments were compared and the results are expressed in Fig. 26. The immunity induced by fused cells remained almost unchanged while the resistance of the animals immunized by the neuraminidase-treated cells tended to decline. The stability of the immune state against further tumor challenges in animals treated with γ-irradiated cells were not certain, as the mice in this group were used for other purposes after the third challenge.

(5) Specificity of Tumor Immunity

Specificity of MC tumor immunity was tested with another tumor (SP) by injecting 10^5 cells into three MC tumor immune mice (resistant to 18 challenges). Tumors developed in these three mice at the site of inoculation at a

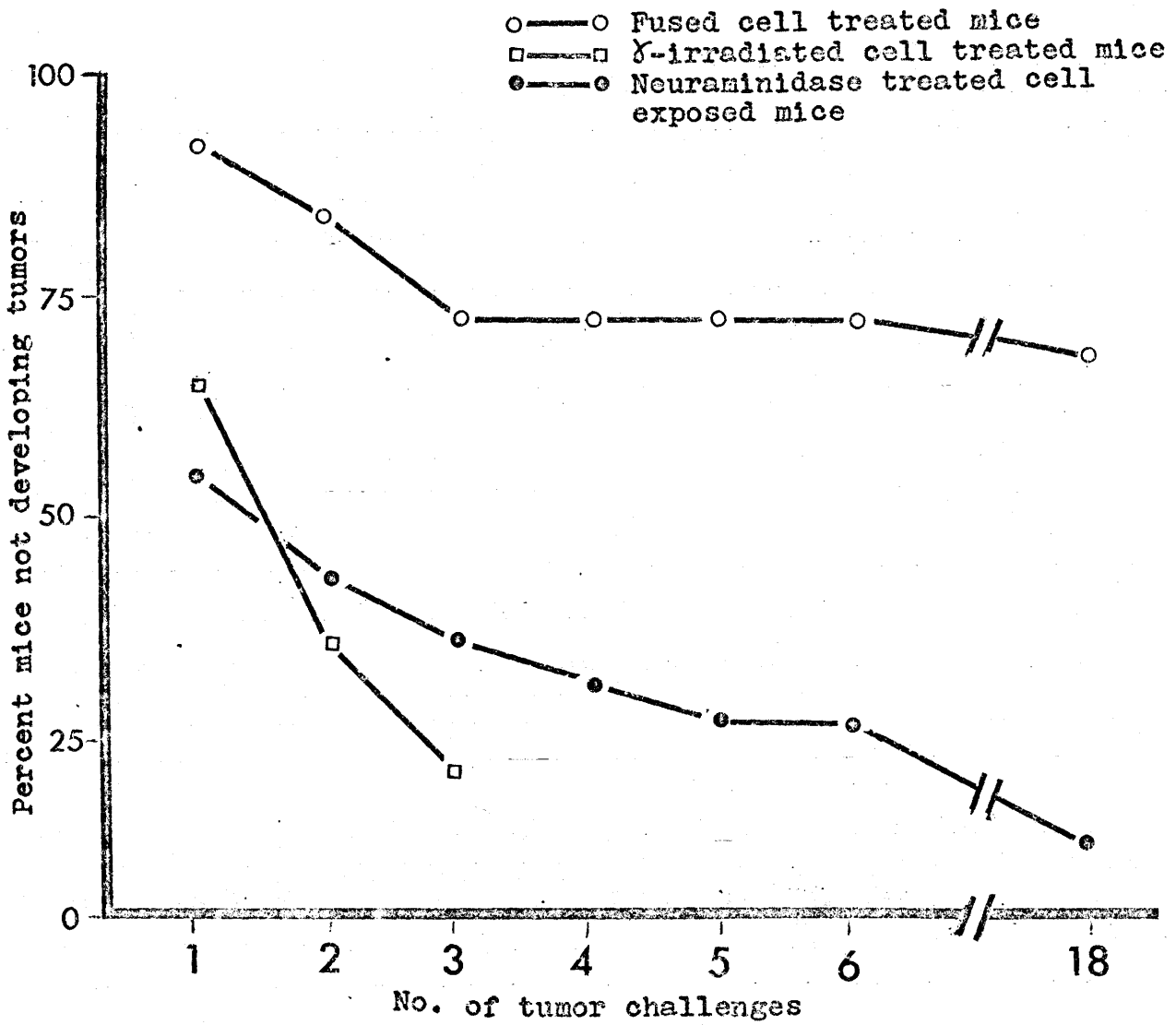

Fig.26. Resistances to repeated tumor challanges in three groups of A/Jax mice immunized with virus fused-, γ-irradiated-, neuraminidase treated- MC tumor cells, respectively.

comparable rate as in the untreated controls injected with SP tumor at the same dose. It indicated that there was no detectable cross immunoreactivity between SP and MC tumors, and the immune state established by fused MC tumor cells was tumor specific.

(6) Histological Picture of the Site of Tumor Rejection

Two immune mice which had rejected 10 tumor challenges were sacrificed 8 days after the last challenge in the left legs. The right had been challenged 37 days before sacrificing the animals. The legs were excised and histologically processed. The sections from the right legs were found completely free from tumor cells. In the sections from the left legs, some areas contained cells with deeply staining cytoplasm and pycnotic nuclei, mingled with lymphocytes and polymorphonuclear leucocytes (Fig. 27). These areas were presumed to be the sites of tumor rejection. It seems that inflammatory processes following cellular immune reactions might be involved in the rejection judging by the histological picture.

Fig. 27. Photomicrograph of a section from a hind leg of a tumor immunized A/Jax mouse showing the site of tumor rejection. The leg was excised and fixed in Davidson's fixative at 8 days after tumor challenge with 1 x 10^5 cells through an intramuscular route. Hemotoxylin and eosin stain. x150.


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III. Controlled Experments

Sendai virus has been shown to exhibit neuraminidase activity (Sokol et al, 1961). The immunogenicity of Sendai virus-fused cells may be due to viral neuraminidase effect on the tumor cells. It is also possible that single cells exposed to Sendai virus (but not fused), may have been responsible for inducing immunity. To test these possibilities, the following experiments were designed. Mice were injected with viable MC tumor cells and UV inactivated Sendai virus in various combinations:

(1) Single unfused cells were separated from fused cells after treatment with Sendai virus in usual fusion and separation procedures. Dosages of 100, 1,000 and 10,000 cells were separately injected into groups of animals. Each group consisted of 5 mice.

(2) Tumor cells and Sendai virus, which had been mixed but without allowing time for fusion to take place, were injected into 10 mice in a dose of 1×10^6 cells.

(3) Tumor cells (1×10^6) were injected into one leg and Sendai virus (552 HAU) in the other leg at the same time. Ten mice were used in this experiment.

The results of the first experiment are summarized in Table VIII. When the mice were injected with unfused free cells at the dose of 10,000, they all developed tumors. However, the survival time of the treated mice were lengthened as compared with those of controls. In the dose of 1000

Table VIII. Fate of single unfused MC tumor cells and their effect on the growth of transplanted tumor in recipient mice

	Pretreatment			Tumor challenge	
	1st	2nd	3rd	1st	2nd
10,000 cells					
single unfused cells	5/5*(43.6)**	-	-	-	-
untreated cells	5/5 (30.0)	-	-	-	-
1,000 cells					
single unfused cells	0/5	2/5(51.6)	3/3	-	-
untreated cells	5/5	2/2***(32.5)	-	-	-
100 cells					
single unfused cells	0/5	0/5	0/5	2/5	3/3
untreated cells	0/5	0/5	0/5	5/5	-

"untreated cells" indicate dispersed MC tumor cells not treated with Sendai virus.
*No. of mice developing tumor/No. of mice tested.
**Figure in the parenthesis represents the mean of survival time (days).
***Two untreated control mice were used only for the second challenge experiment as controls in the indicated group.

cells, no tumors resulted, while the controls developed tumors when injected with untreated cells at the same dose. Subsequently, similar doses of virus-treated single cells were given again to the animals as in the usual tumor immunization course previously described. In this treatment, 2 out of 5 mice gave rise to tumors, and 3 other mice finally also grow tumors on the third occasion of the treatment. In the third case, all 5 mice were free from tumor development after three injections with 100 virus-treated-single cells. No tumor grew either in the 2 control mice injected with 100 untreated cells. Tumor challenges with 1×10^5 cells were performed in these mice. In the experimental group, 3 were resistant to the first challenge. However, these resistant animals developed tumors on the second challenge. The control mice, which were given 100 virus-treated single cells after the first challenge, developed tumors at the site of injection.

In two other experiments, cells mixed with virus but without allowing time for fusion, and viable cells and virus injected into opposite legs, produced tumors on the first occasion of the treatment. The latent periods for tumor appearance in both experimental groups were longer than those of the controls (both the second and third experiments in comparison to the respective controls, $p<0.001$). However, the survivals of both experimental groups were not statistically different from the control groups. In each experiment, two control animals were injected with corresponding amount of untreated tumor cells and all developed tumors.

IV. Delayed Hypersensitivity

It was important to determine whether delayed type of cutaneous hypersensitivity reactions could be elicited by tumor antigens in the present immune system. It was also necessary to find out whether these would follow the pattern observed for specific transplantation resistance. Six actively immunized mice (immune from tumor challenges three times) were the first to be tested for sensitivity to the immunizing tumor. The test tumor antigens were prepared by tumor fragments in 0.9% NaCl (20% W/V) in a homogenizer. The homogenate was centrifuged and the supernatant was used for the test. 0.1 ml. of this solution was injected intradermally into the left hind foot-pad and at the same time equal amounts of normal saline were injected in a similar way into the right hind foot-pad of the 4 immunized mice. Another two immunized mice were injected with 0.1 ml. of a mixture of the supernatant of tumor homogenate and complete adjuvant (1:1, V/V). No detectable swelling was observed in either foot in the immunized or control mice by the treatment described.

Various forms of tumor antigens were prepared and used for foot-pad testing, since the supernatant of tumor extract did not demonstrate positive reactions on the foot-pads of immune animals. Preparations of four different concentrations (1×10^3, 1×10^4, 1×10^5 and 1×10^6 cells/ 0.1 ml.) of viable single tumor cells and frozen and thawed-tumor cells were tested in a similar way as described previously.

Eight animals, which were immunized by fused cells and then resistant to two tumor challenges, were used in this experiment. It was 12 days after the second challenge when the experiment was performed. The results are shown in Table IX. Only live tumor cells at higher concentrations (1×10^5 and 1×10^6) were effective for a positive reaction. This consisted of marked swelling of the affected foot-pad 18 to 24 hours after injection (Figs. 28 and 29). The viable cells at concentration of 1×10^4 gave an equivocal result. Disrupted (frozen and thawed) tumor cells at 4 different concentrations, 1×10^3 viable cells, or physiological saline failed to produce positive reactions in the immunized mice. All normal control animals given either viable cells, disrupted cells or physiological saline did not confer positive reactions on the foot-pads. However, the normal mice received viable tumor cells and all developed progressive tumors at the site of injection. 5 to 8 days after inoculation.

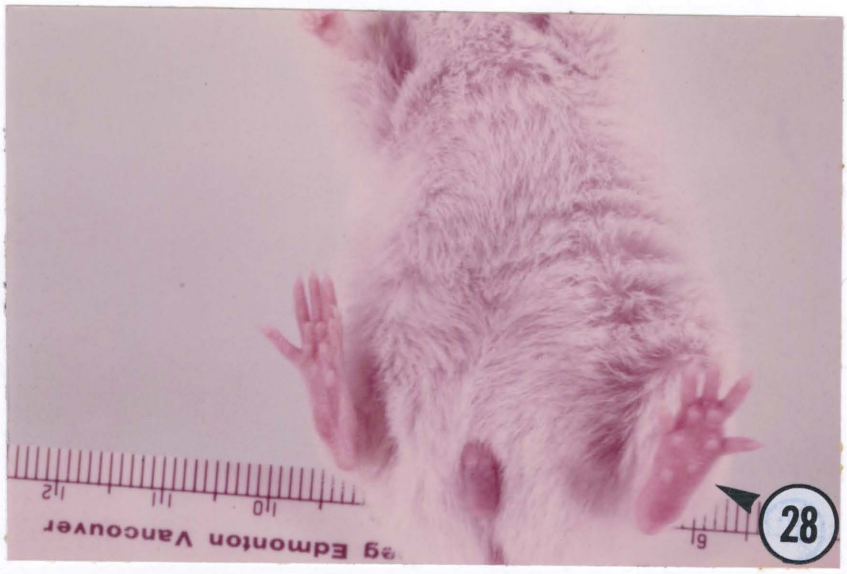
The positive results of delayed hypersensitivity considered in the above experiment were supported by the histological appearance of the reaction. In the immunized mice demonstrating positive reactions at 24 hours, hematoxylin and eosin stained sections from the tissue of foot-pads showed edema and a prominent cellular infiltration (Fig. 31). The cellular components of the reaction consisted of mononuclear lymphoid cells and a few polymorphonuclear leucocytes (Figs. 32 and 33). Some tumor cells could also be recognized in the

Table IX. Effectiveness of delayed type (24 hours) hypersensitivity reaction of mice immunized with fused cells and of normal mice to footpad injection with various forms of tumor

Form of tested tumor preparation	Reaction		Remarks
	Sensitized mice	Normal mice	
Fresh, single viable tumor cells			
1×10^3	-	-	Tumor developed at the site of injection in sensitized and normal mice
1×10^4	±	-	"
1×10^5	++	-	Tumor did not develop in sensitized mice, but took in normal control mice
1×10^6	+++	-	"
Frozen and thawed, 3x disrupted tumor cells			
1×10^3	-	-	Tumor did not develop at the site of injection
1×10^4	-	-	"
1×10^5	-	-	"
1×10^6	-	-	"

+++ 0.81-1.00 mm.; ++ 0.61-0.80 mm.; + 0.41-0.60 mm.; ± 0.21-0.40 mm.; - <0.21 mm.

Figs. 28 and 29. Foot-pad reactions in the MC tumor immunized (by fused cells) male A/Jax mouse, 24 hours after cutaneous injection. Both pictures were taken from the same mouse. The right foot-pad was given physiological saline alone, while left foot-pad (arrow) was given 1×10^6 viable MC tumor cells.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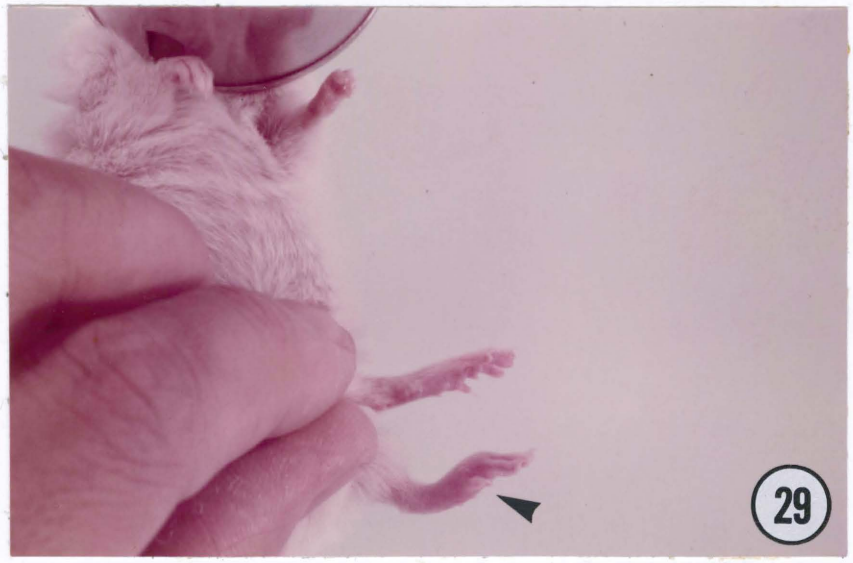
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Figs. 30 & 31. Foot-pad reactions in the MC tumor immunized mouse, 24 hours after cutaneous injection. Hematoxylin and eosin stain. x 140.

Fig. 30. Control, given injection of physiological saline alone at the right foot.

Fig. 31. Given injection of 1 x 10^5 viable MC tumor cells.


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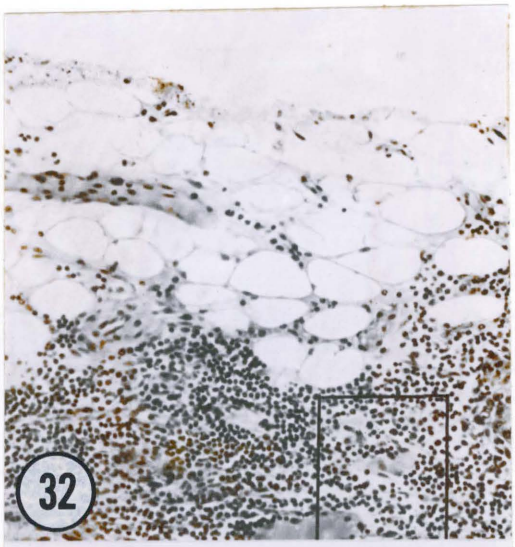
Figs. 32 & 33. Photomicrographs of a section from the foot-pad of an immunized mouse, 27 hours after cutaneous injection with 1 x 10^5 viable MC tumor cells. Hematoxylin and eosin stain.

Fig. 32. Mononuclear cell infiltration in the area of delayed hypersensitivity reaction is evident. x80.

Fig. 33. Part of the previous micrograph was enlarged. x 400.
Note clearer picture of the area filled with mononuclear and polymorphonuclear leucocytes. Several lymphoid cells appear to be in mitosis (small arrow) and several tumor cells (large arrow) could vaguely be recognized in the area.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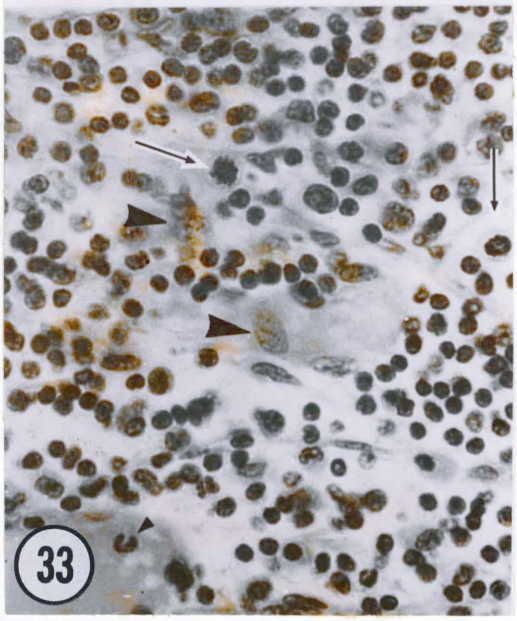
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area. In the other foot-pads injected with normal saline, the reaction was considered negative, as edema and cellular reaction were minimal (Fig. 30).

As timing and appearance of the reactions are both characteristics of delayed hypersensitivity, and certain time interval is needed for the host resistance to build up, experiments were designed to find out a maximum immune response of the host animals after immunization with fused cells. This was tested by injecting viable tumor cells into foot-pad. Nine immune mice following the third tumor challenge were used in this experiment. The mice were given 1×10^5 live MC tumor cells at 5, 10 or 20 days after the last tumor challenge. Observations were made at 6, 24 and 48 hours after inoculation. This increase in foot-pad thickness was calculated by comparing the thickness of the tumor injected and saline injected foot-pads on each occasion. As illustrated in Fig. 34, very slight reaction (14% increase in thickness) at 24 hours occurred in mice 5 days after challenge (resensitization). Delayed hypersensitivity reaction with 42% and 26% increases in thickness of foot-pads at 24 hours after inoculation were observed in mice tested 10 and 20 days after challenge, respectively. It is apparent that the strongest reaction in mice tested was the one 10 days after challenge.

Another series of tests were carried out to determine whether active immunity induced was specifically against the immunizing MC, and to determine the reactivity of tumor-bearing-

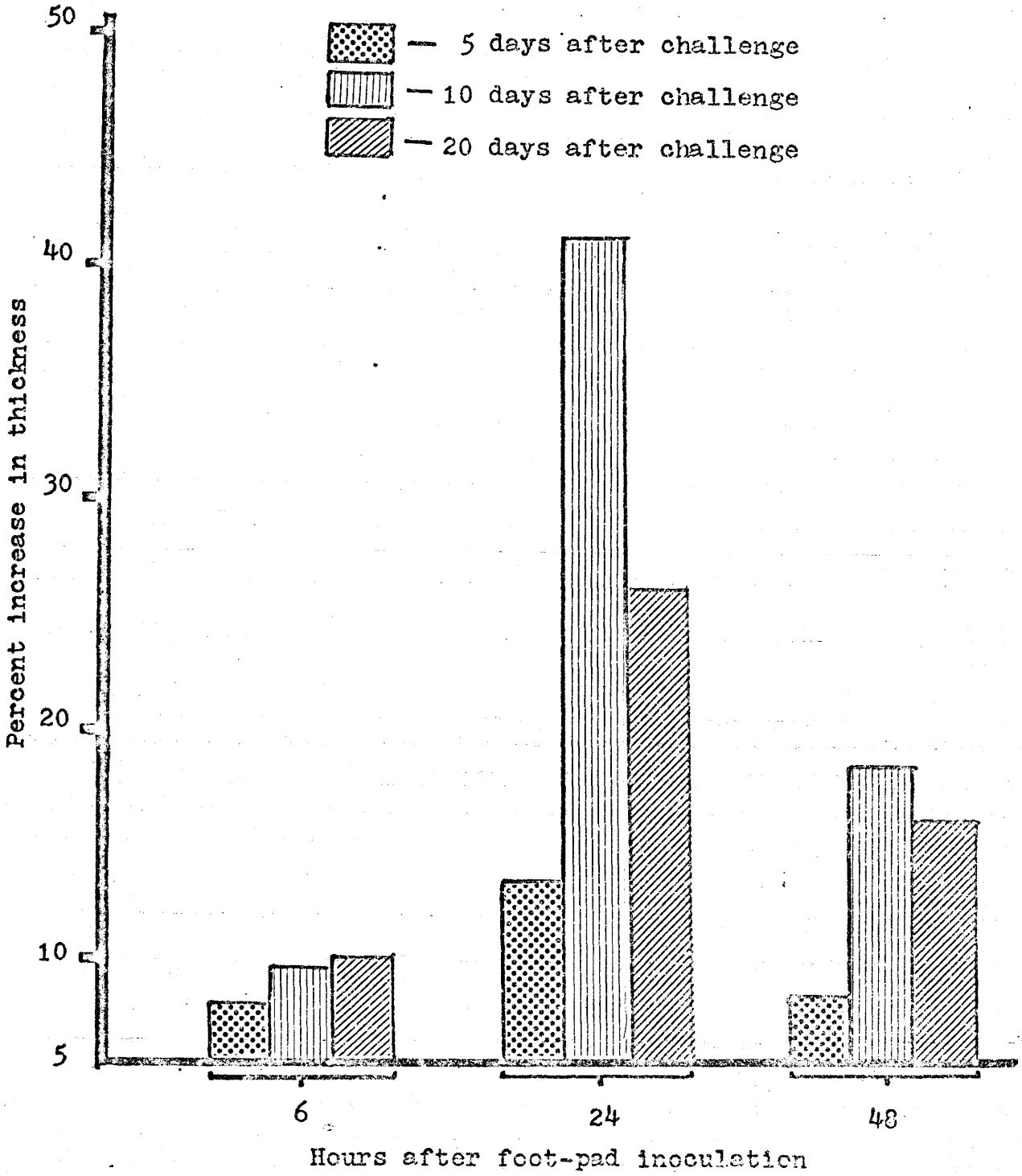

Fig. 34. Foot-pad delayed hypersensitivity to MC tumor inoculation in actively immunized A/Jax mice at various days after the second challenge doses were given.

mice to the foot-pad injection of MC tumor. Sixteen mice, 8 immunized animals 10 days after the third challenge, 4 MC tumor-bearing (10 days after tumor inoculation) and 4 untreated mice were used in this experiment. The results are presented in Table X. Immunized mice, except for one (#8), exhibited positive reactions of varying degree at 24 hours after injection of 10^5 viable cells on footpads. However, the reaction of the #8 mouse, in which the third challenge tumor was not overcome and just became palpable at the time of the test, proved to be negative. The other hind foot-pads injected with normal physiological saline showed negative responses. The delayed hypersensitivity reaction did not occur on either foot-pad in tumor-bearing or normal mice.

Except for #8, the immunized mice all rejected the tumor cells injected into the foot-pads. However, the normal and tumor-bearing mice showed swelling of the injected feet at day 5 to 7 and progressively growing tumors resulted.

In addition to the above experiments, 2 immunized mice were tested with another tumor (SP). Negative results were obtained, since no pronounced swelling could be detected at 24 hours. Nevertheless, tumors developed at the sites of injection and eventually caused the death of the animals. These results confirm that the immune response induced by the fused cells was immunizing tumor specific.

Table X. Delayed hypersensitivity tests

Immunized mice (from expt #12)					Control mice				
Increase in foot pad thickness (in mm.)					Increase in foot pad thickness (in mm.)				
	Viable tumor cells		Physiological saline			Viable tumor cells		Physiological saline	
Mouse #	24 Hrs	48 Hrs	24 Hrs	48 Hrs	Mouse #	24 Hrs	48 Hrs	24 Hrs	48 Hrs
1	++++(1.04)	++(0.82)	-(0.16)	-(0.07)	1**	-(0.17)	-(0.15)	-(0.15)	-(0.07)
2	++(0.78)	±(0.39)	-(0.09)	-(0.04)	2**	-(0.13)	-(0.09)	-(0.17)	-(0.05)
3	++++	+	-	-	3**	-	-	-	-
4	+++	+	-	-	4**	-	-	-	-
5	++	+			5***	-(0.10)	-(0.12)	-(0.15)	-(0.08)
6	++	+			6***	-(0.13)	-(0.10)	-(0.10)	-(0.03)
7	++	±			7***	-	-	-	-
8*	±(0.27)	-			8***	-	-	-	-

++++>1.00 mm; +++ 0.81 - 1.00 mm; ++ 0.61 - 0.81 mm; + 0.41 - 0.60 mm; ± 0.21 - 0.40 mm; -<0.21 mm.
* Tumor became palpable at the time of the test (10 days after 3rd challenge with tumor cells).
** Mice #1-4 tumor bearing mice (10 days after tumor injection).
*** Mice #5-8 untreated control mice.

V. Adoptive Transfer of Tumor Immunity

This series of experiments was undertaken to see if immunity of MC tumor could be adoptively transferred by lymphoid cells from fused cell immunized animals to normal syngeneic hosts. Spleen cell and lymph node cell suspensions were prepared from immune mice. Each of resulting spleen or lymph node suspension, or mixture of spleen and MC tumor cells was injected into a group of 5 to 10 virgin animals. Three experiments were performed.

In the first experiment, 5 groups, each consisting of 10 mice were used as recipients. To obtain immune spleen cells, 50 immune mice which had been resistant to at least 11 tumor challenges were sacrificed. One minced immune spleen was transferred to each recipient. The time interval between spleen transfer and tumor challenge (S-T) in the recipient animals was -2 days, 6 days, or 12 days. With exception of -2 days of S-T where the spleens were transferred two days before tumor challenge, spleen cells in other conditions were all transferred at the indicated days after tumor administration of 1×10^5 cells.

In the second experiment, 4 groups, each containing 5 mice, were used as recipients. Mixtures of immune spleen and tumor cells in 100:1, 50:1, 10:1, and 1:1 ($\times 10^5$ cells for each) splenic lymphoid cells to tumor cells ratio were injected intraperitoneally into each group on day 0. Ten control animals for these above two experiments were injected with 1×10^5 viable tumor cells on day 0.

The results of the first and second experiments are summarized in Table XI. As only one group of controls was used to compare 9 groups of treated animals, the difference of treated vs. control animals with regard to survival was tested by Dunnett's method, after logarithmic transformation was used to stabilize the variance. In both experiments, no resistance to tumor development was observed in terms of complete tumor rejection. However, in the first experiment, the survivals of the recipient animals with S-T intervals of -2, 2 and 6 days were statistically longer than that of controls at significant levels of 5%, 1% and 5%, respectively. It, therefore, appears that animals which were given immune spleen cells two days after tumor challenge conferred a higher protection.

In the second experiment when mixtures of immune spleen cells and tumor cells in various proportions were injected with groups of virgin mice, mean survivals in treated group were apparently increased as the spleen:tumor cell ratio increased. Among them, only the group injected with a mixture of spleen and tumor cells in proportion of 100:1 was found to be statistically significant at the 5% level as compared with the control group; although the third treatment (50:1) was approaching the significant level.

On basis of these results, it seemed that some degree of immunity was transferred to the recipients, but it did not reach the level at which the animals could suppress the tumor growth. What could be done to enhance it? (i) A more prolonged and repeated course of immunization. (ii) Smaller challenge inocula might serve to demonstrate smaller degrees of immunity. The

Table XI. Adoptive transfer of immunity
Experiments 1 and 2[a]

Group	No. of Animals tested	Mean survival ± S.E. Days	(Log_{10} transformation)	t value
Control	9	28.56 ± 1.51	(1.45 ± 0.03)	
Experiment 1 S-T intervals (days)				
-2	9	33.78 ± 0.76	(1.53 ± 0.01)	-2.70*
2	9	35.11 ± 1.46	(1.54 ± 0.02)	-3.20**
6	10	33.10 ± 0.86	(1.52 ± 0.01)	-2.44*
12	9	28.44 ± 1.56	(1.45 ± 0.02)	0.02
Experiment 2 Splenic lymphoid: tumor cell ratio ($\times 10^5$)				
1 : 1	5	28.60 ± 1.63	(1.45 ± 0.02)	-0.12
10 : 1	5	32.60 ± 2.29	(1.51 ± 0.03)	-1.72
50 : 1	5	33.60 ± 3.29	(1.52 ± 0.02)	-2.01
100 : 1	5	34.80 ± 1.24	(1.54 ± 0.02)	-2.65*

[a] Nine treated groups (Experiments 1 and 2) were compared to one control group of mice with regard to animals' survivals by Dunnett's test.
Analysis was based on log10 transformation of the data.
*Significance at 5% level.
**Significance at 1% level.

smaller challenge inoculum used should be that which produced above the threshold dose in control animals. With these considerations in mind, the third experiment was designed, although this time immune lymph node cells were used instead of immune spleen cells. Fifteen mice were injected intraperitoneally with 2×10^6 minced regional lymph node cells from immune mice on three occasions at 6-day intervals. Ten days after the lymphoid cell transfer, 15 mice were divided into three groups (5 mice in each) and challenged with 1×10^4 and 1×10^5 live MC tumor cells.

The results shown in Table XII indicate all treated animals challenged with 1×10^5 tumor cells developed tumors and a high protective effect was induced in the treated mice which were challenged with lower inocula. There was only one mouse developing a tumor in the group of mice challenged with $1/2 \times 10^5$ cells. No tumors developed in the group of mice challenged with 1×10^4 cells. The mice in the control groups all grew tumors after being challenged with various tumor doses. The tumor in the only treated mouse which failed to reject the challenge became palpable 10 days after tumor was injected while the controls did so at about 6 days. The survival of this mouse was 48 days after challenge (control mice: average 29 days).

VI. Splenic Lymphoid Cellular Responses in vitro after Immunization

In order to explore immune state of the immunized mice in relation to lymphoid activity and reactivity to PHA, the following experiments were performed.

Table XII. Experiment 3
Adoptive transfor of immunity

Tumor challenge dose	Mice developing tumors/total mice tested	
No. of cell	Experimental	Control
1×10^5	5/5	2/2
2×10^4	1/5	1/1
1×10^4	0/5	2/2

The experimental group of mice were injected intraperitoneally with immune lymph node cells on three occasions before tumor challenge.

Splenic lymphoid cell preparations were studied from 5 immune mice and 5 normal control mice of comparable age. The immune mice had been given three separate fused cell immunizing doses and were resistant to one tumor challenge. The spleens were removed from the immune mice 15 days after the challenge. The proliferative activity of lymphoid cells assayed by ^{3}H thymidine incorporation is shown by the results of experiments (Table XIII). The results were expressed as mean radioactive counts per minute (cpm) per culture containing 2 x 10^{6} lymphoid cells for each spleen of triplicate cultures. There was good agreement between the individual radioactivity levels within each triplicate, usually on the order of 5-10%. However, a wide range of variations in the radioactivity was found in individual within each group especially among those from immune mice. The unequal error variance reflects individual variation. The data were required to be transformed to logs to stabilize the variance and achieve additivity. As a result of this transformation, the standard deviation varies directly as the mean. A one way analysis of variance was done for the control versus the immunized group, ie, to make comparisons at each of the three levels separately. A final two way analysis of variance was then done on all the data.

The control splenic lymphoid cell culture without adding PHA showed a low level of radioactivity. However, under the parallel condition immune lymphoid cells exhibited a high rise in radioactivity, this being 23 fold greater than the control (the difference is significant at 1% level). Since input cell concentration in cultures was the same in both immune and control groups, these observations indicate that a high degree of

Table XIII. Tritiated thymidine incorporation by normal and immune A/Jax mouse spleen lymphoid cells with or without PHA stimulation in culture

Experimental group	^{3}H-thymidine incorporation (mean cpm/culture) ± S.E.			Ratio of incorporation	
	unstimulated	PHA stimulated 1:25	PHA stimulated 1:250	PHA 1: 25 stimulated/ unstimulated	PHA 1:250 stimulated/ unstimulated
Control	95 ± 17	4,933 ± 706	1,932 ± 261	51.9	20.3
	(2.45 ± 0.22)[a]	(3.74 ± 0.07)	(3.46 ± 0.08)		
Immune	2,211 ± 1,548	7,248 ± 1,877	4,884 ± 896	3.3	2.2
	(2.95 ± 0.28)	(3.80 ± 0.72)	(3.65 ± 0.10)		
Ratio of incorporation Immune/Control	23.3	1.3	2.5		
Difference (t value)	3.45**	0.95	3.31*		

In these experiments, 2 x 10^6 spleen lymphoid cells were cultured in 2 ml. of medium with or without PHA for 48 hours of culture. ^{3}H thymidine was added for the final 5 hours of culture. Results are expressed as the mean counts per minute of five animals in each group having triplicate samples for each spleen in culture. The triplicates in each spleen culture have less than 10% variation in counts

a Figures in parenthesis indicate mean cpm/culture ± S.E. after $\log_{10}(X)$ transformation.
** Significance at 1% level; * significance at 5% level.

spontaneous DNA synthesis of lymphoid cells occurred in the immune mice, at least in the spleens. This is compatible with an ongoing immune response in the immune animals in which viable tumor cells were injected 15 days before the test.

The response to stimulation by PHA was tested in spleen cells from immune and control animals. The PHA was given at two dose levels; 1/25 which was donsidered as an optimal dose and one lower (1/250) for stimulation in mice. Using control lymphoid cells, ^{3}H-thymidine incorporation was increased more than 50 fold with the optimal dose and more than 20 fold with a lower concentration of PHA. The equivalent figures for lymphoid cells from the immune animals, in contrast, were about 3.3 and 2.2 times the levels found in unstimulated immune spleen cell culture. A significant difference between the immunized and control groups was demonstrated at 5% level in response to 1:250 PHA, but not to 1:25 PHA stimulation. However, it should be remembered that spontaneous DNA synthesis was high in lymphoid cells from the immune animals in unstimulated culture.

For the two way analysis of variance, ie, bringing together all the data, significant differences were demonstrated between the controls and immunized groups, and between the three levels PHA. No significant differences were shown to exist among mice, nor were the interactions between level and preparation present.

During the course of the splenic cell preparation before setting up the cultures, a high degree of cell agglutination was noted in the spleen cells obtained from the immune animals, but not in those from the control normal animals.

VII. Anti-tumor Agglutination

Using the tube and slide agglutination techniques, high agglutinating activity was detected in the sera of all of 5 immune mice used in the previous experiments (VI). Titers ranged from 1:1280 to 1:5280 (Table XIV). In the sera of the control animals, no agglutination was detected above 1:20.

VIII. Demonstration of Tumor Specific Humoral Antibody

1. Immunofluorescence Studies

Samples of serum, obtained 18 days after the third tumor challenge, from two immune mice were tested for the presence of anti-MC tumor antibody. Each immune serum preparation was tested on frozen sections of the fresh MC tumor, SP tumor, and liver from a normal A/Jax mouse. The latter two tissues served as controls. When the sections were examined microscopically under ultraviolet illumination, bright fluorescence was observed on the cells of MC tumor sections when immune serum was used. The fluorescent dots were located primarily on cell periphery and some in the cytoplasm of tumor cells. A very little fluorescence was noted in the nuclei. The cells in the necrotic areas were not distinctively stained. No appreciable fluorescence was observed on the section of SP tumor nor of the mouse liver. The reactions of the sera were similar from both immune mice used in the test.

Sera from the two normal mice were tested under similar conditions and no fluorescence was noted on the sections of

Table XIV. Aggulutination of MC tumor cells by immune and normal mouse sera*

Mouse #	Immune serum	Normal serum
1	1:1280	1:20
2	1:5210	1:8
3	1:2560	1:16
4	1:1280	1:8
5	1:5210	1:4

* Duplicate experiments were performed for each test. Titer is the highest dilution of serum showing clearcut aggulutination.

MC tumor, SP tumor or mouse liver.

The specificity of serum antibody to MC tumor was also determined by the absorption studies. Incubation of antiserum obtained from one of the MC tumor immune mice (10^8 viable MC tumor cells/ml. serum) at 0°C for one hour to absorb the antibody. The serum was then tested and no positive staining was observed with MC tumor on the sections.

To determine whether there was circulating anti-MC tumor antibody in the MC tumor-bearing mice, sera of two 18 day tumor-carrying-mice were tested. No fluorescence was seen on the frozen sections of MC tumor, SP tumor or mouse liver.

2. Effect of Serum from Tumor Immune Mice on the Growth of Transplanted MC Tumor

In two experiments, after MC tumor cells were incubated with MC immune serum the tumor growth was markedly enhanced in syngeneic mice as compared with controls. The controls were treated with normal mice serum or untreated with serum (Table XV). The enhancement was revealed by a shorter latent period of tumor appearance and an advenced growth rate of cells treated with the tumor specific antiserum. The mean tumor diameters at 20 days in mice treated with anti-MC tumor serum undiluted and 1:25 diluted were significantly greater than those in normal serum treated controls ($p < 0.01$ and < 0.05 in experiments 1 and 2, respectively).

Table XV. Effect of Tumor Specific Isoantiserum on Tumor Growth in Syngeneic Recipient A/Jax Mice.

Experiment	Serum Dilution*	Mice Developing Tumors/Total Mice Tested	Mean Latent Period (Days)	Mean Tumor Diameter (Mm) After 12 days	20 days
	non serum treated	2/2	7.0	11.5	20.5
1	normal, undiluted	6/6	6.3	13.3	23.4
	immune, undiluted	6/6	4.8	17.5	36.1
2	normal 1:25	6/6	6.6	10.9	21.6
	immune 1:25	6/6	5.7	14.7	31.5

Serum and tumor cells were incubated at 37°C for 40 minutes before intramuscular injection into animals.

*PBS was used as diluent.

DISCUSSION

For transplantation, the tumor was prepared as a suspension containing dissociated cells. The neoplasm regularly produced 100% take when only 400 viable cells were injected into the mice. The specificity of the MC sarcoma, as received from Dr. Buck, was demonstrated by its failure to grow in all but its indiginous strain, A/Jax mice. The tumor appeared to be host specific for A/Jax, since it has been tested in strain A mice obtained from Houston and five other mouse strains in which it failed to develop tumors.

Studies on chromosomes of the MC tumor over a period of 2 and half years invariably showed a modal chromosome number of 43, including always one and occasionally two metacentric marker chromosomes. In general, chemically induced tumors are individually distinct as regard to antigenic specificity, in contrast to the fact that different tumors induced by a given virus show an extensive and possible complete antigenic cross reactivity. The immunizing capacity of methylcholanthrene induced tumor normally persisted a long period of passages in vivo. It must be admitted, however, that MC tumor which has been transplanted serially for long periods of time probably differs from the primary tumor. It has been shown that in some instances, the tumor antigenicity appeared to diminish upon serial transplantation in syngeneic hosts (Klein and Klein,

1962). This was explained as being due to a selection of less antigenic cells or antigenic simplification. On the other hand, there is also the possibility of acquisition of new antigens in transplanted tumors through repeated passages, resulting in stronger antigenicity. For instance, the transplanted tumor might have contained isoantigens of the primary tumor host, yet some of these isoantigens might have been lost from animal strains (by mutation) during continued inbreeding. In such circumstances, the tumor cells remain or become antigenic even if the original TSTA's have diminished, since the isoantigens in the tumor cells would manifest themselves as the TSTA's which would be the only antigenic difference between the host and tumor. Alternatively, the tumor cells might change genetically in the course of serial passages in the same inbred host, thereby gaining antigens. It cannot be stated with certainty that the tumor has not been contaminated with viral materials, even though no viral particles were detected in the tumor by the electron microscopic observations. Instances of antigenic loss as well as antigenic gain have been discussed (Green, 1959; Witebsky, 1961; Tennant, 1970). No matter what the situation as far as antigens are concerned, the tumor remained specific to its syngeneic host and we believe that it is worthwhile to test anti-tumor immunogenicity in such an animal host-tumor system. In the interpretation of the results, one must assume that the chemically induced tumor used in this

study may not necessarily be immunologically representative of chemically induced tumors which are of recent origin (Riggins and Pilch, 1964; Old et al, 1962; Prehn, 1963b).

Attention has recently been paid to expression of malignancy in various hybrid cells. Barski and Cornefert (1962) demonstrated the dominant character of hybrid cells which arose spontaneously in mixed cultures of a highly malignant mouse lines. A similar observation has been made by Scaletta and Ephrussi (1965). Hybrids between tumor and normal mouse cells produced tumors in the same way as the malignant parent cells. In contrast to these results, Harris and coworkers (1969) have shown suppression of malignancy by cell fusion between malignant and nonmalignant cells. Of great interest in tumor immunity along this line is that the hybrids between Ehrlich ascites and hamster transformed cells have been shown to lose their tumorigenicity (transplantablity). The hybridization induced immunity against Ehrlich ascites cells when these are subsequently introduced into the mice (Watkins and Chen, 1969). The introduction of the Sendai virus cell fusion technique has rendered it possible to fuse almost any mammalian cells (Okada et al, 1957; Harris et al, 1966). Despite the current interest of cell fusion in various biological fields, little attention has been paid to the loss of transplantability by fused tumor cells of the same origin or type, although the fate of such fused cells was pointed out 14 years ago: "...

it is certain that the fused cells undergo degeneration at last" (Okada et al, 1957). Although the actual mechanisms of cell fusion were still unknown, it was suggested that virus might alter the structure of the cell periphery, involving the configuration of either lipid or protein micelles or both, initially by binding to it (Roizman, 1962; Lucy, 1970). The changes might well be expected to be reflected in abnormal cellular behavior.

It has been shwon by Okada and associates (1957) that fusion required viable cells as Ehrlich ascites cells aggregated following injection with Sendai virus and degenerated cells were rejected from cell aggregates. This also could be inferred from the experiences of the present study that a considerable increase in the percentage of dead cells was found among the single cells after fusion. Technically, we therefore used the cells with or over 80% viability (live cells per total cells) for fusion. Ho and Gorbunova (1962) indicated that fusion activity occurred in most cell types of human and animal origins examined and yet it varied in amount with different viruses. They further noted that the fusion activity parallelled HA titer but was not identical with it. Okada and Todokoro (1962) found that in Ehrlich ascites tumor cells and Sendai virus, fusion factor of the virus was different from its infectivity, HA activity, and was not related to its neuraminidase activity.

Sendai virus-fused multinucleate MC tumor cells lost their transplantability and stimulated a protective reaction in

the recipient mice. Twelve separate experiments were performed in mice by injecting three immunizing doses of fused cells and the results indicate the bodys' immunological defenses could be built up against challenges with viable tumor cells (Table V). The control group showed a 100% progressive increase in tumor size until death of the animals. Injection of either γ-irradiated tumor cells or neuraminidase treated tumor cells were compared with the present immunizing method in respect to the strength of immunogenicity. As determined by three subsequent tumor challenges, it was shown that fused cell treated mice attained a striking resistance (73%), whereas the resistances induced by γ-irradiated cells and neuraminidase treated cells were 20% and 37% respectively. During the course of repeated tumor challenges, the immunity produced by fused cells appeared relatively persistent, while the mice immunized by neuraminidase treated cells tended to decline in their resistance (Fig. 2). The stability of the immune state in γ-irradiated cell exposed mice is not clear, as the mice were used for some other purpose and not tested after the third challenge. It should be kept in mind that when the tumor for challenge was inhibited to grow in immunized mice, it usually regressed, since its presence in the body also reinforced the preexistent immune state. On the analogy of the efficacy of attenuated microbial vaccine, it does seem that virus fused cells have immunizing powers better than tumor cells treated by chemical or physical means.

The specificity and an immunological basis of the protective reaction seem evident for the following reasons: (i) Immune animals had no protection against the growth of a spontaneous tumor (SP) of A/Jax mouse. (ii) Positive results for delayed type hypersensitivity were consistently shown in the immune mice, accompanied by subsequent rejection of the tested tumor inoculum. Negative results were obtained when SP tumor was tested in the same way. (iii) Positive fluorescent staining reaction was demonstrated with only MC tumor, but not with SP tumor, using antiserum obtained from the immune mice. (iV) Immune sensitivity in respect to MC tumor could be adoptively transferred to normal virgin animals by means of living lymphoid cells from the regional nodes of actively immunized animals. (V) A high degree of spontaneous DNA synthesis of the lymphoid cells obtained from the spleens of immunized animals was demonstrated in vitro.

A completely different approach to immunotherapy has been developed by Czajkowski and associates (1966, 1967), who have shown that antigenicity of the tumor cells could be enhanced by coupling them to an antigenic protein carrier (e.g. human or rabbit gamma-globulin) with bidiazobenzidine. Results in animals or in patients injected with this complex are encouraging and suggest that both cellular and humoral immunity are activated and directed against the tumor cells. In the investigations of cells for the attachment of myxoviruses, viral coated-protein molecules, namely hemagglutinins, have

been found to incorporate into the plasma membrane of virus exposed cells (Hotchin et al, 1958; Morgan et al, 1962; Marcus, 1962). The sequence of events of such surface conversion are that cellular receptors have to be attacked by viral destroying enzyme (specifically neuraminidase) before viral lipoproteins become incorporated into the host membranes. As far as the present immunizing technique is concerned, it is difficult at present to fully explain the mechanisms of fused cell action in immunogenecity. However, it appears clear that during tumor cell fusion by Sendai virus the coat components of the virus would become incorporated into cell membrane. Although the modification of cell surface in terms of molecular configuration has still not been understood, the viral lipoproteins might well be bound to the TSTA's in some way within the cell membrane. The modified fused cells are merely attenuated, in the sense that their growth rate is slowed down or they lose their transplantability, and/or render themselves more immunogenic in isogenic hosts. This is further supported by the results that the failure of immunoprotection by viral treated single cells with varying doses in the controlled experiments. The rationale of the present experiments has some similarity to Czajkowski's study (1966,1967) previously mentioned and also to the concept of "artificial heterogenization of tumor specific antigens" by Russian investigators (Svet-Moldavsky and Hamberg, 1964, 1967; Hamberg and Svet-Moldavsky, 1967). The same principle was recently interpreted by Mitchison

(1970). He suggested that a helper determinant, which can be hapten, a protein, a viral coat protein or a xenogenous cell antigen is introduced into a population of tumor cells. An immunological reaction occurs against the helper determinants after transplantation of the modified tumor cells. As result of this, the reaction to the accompanying TSTA's of the tumor cells is enhanced.

The possibility of direct viral oncolytic effect and the immunological oncolysis was proposed by many investigators (Moore, 1960; Lindenmann and Klein, 1967; Webb and Smith, 1970). Viral oncolysis could be excluded in the immune reaction elicited by the fused cells, as the virus used has been inactivated.

Interferon was shown to be induced by certain viruses including Sendai virus even when UV-inactivated. If a tumor is induced by a virus, infection of the tumor-carrying-host with a second virus might interfere with the growth of tumorigenic virus or the transformed cells through interferon induced by the second virus. It has been demonstrated in several cases, such as Friend virus leukemia in mice that progression can be inhibited by infection with Sendai virus (Wheelock, 1966). Also administration of an interferon preparation delayed the evolution of Friend and Rauscher leukemia in mice (Gresser et al, 1969). Mouse interferon was also effective in increasing the survival of mice bearing chemically induced tumor which was contaminated with intracellular type A "viral particles". Recently the suppression of tumor growth in animals inoculated with interferon inducers has also been

described (Levy et al, 1969). By electron microscopy, there was no indication of the presence of any virus particles in the MC tumor used in the present study. It seems unlikely that interferon produced by irradiated Sendai virus might play a significant role in immunoprotection in the present system. Sendai virus neither protected the animals from the growth of tumor cells nor prolonged the animals' survivals when 1×10^6 single tumor cells were injected into one leg and virus suspension (522 HAU) in the other at the same time.

It has been shown that many tumor cells possess a mucoprotein coat and digestion of this layer with neuraminidase, demonstrated the presence of sialic acid (Gasic and Gasic, 1962; Weiss, 1966). The high negative surface charge on malignant cells has been considered to explain their failure to exhibit normal contact inhibition of movement (Abercrombi and Ambrose, 1962). A hypothesis advanced by Currie and Bagshawe (1967) was that the free carboxyl group of sialic acid in pericellular sialomucin confers a strong electronegative effect which repels negatively charged lymphocytes. This masking of the cell periphery by sialic acid was considered to be analogous to the demonstration that humoral antibody can protect (or inhibit) antigenic sites from immune lymphoid cells' recognition and interaction. On the basis of this concept, Currie and Bagshawe further demonstrated significant immunoprotection in mice after injection with neuraminidase treated tumor cells of various types (Currie and Bagshawe, 1967, 1968a&b, 1969; Bagshawe and Currie, 1968). In the

course of the studies of viral oncolysis, Lindenmann and Klein (1967) have shown that injection of neuraminidase (between 150 µg and 600 µg) along with Ehrlich ascites tumor actively prevented the animals from developing tumor in most A2G mice. However, Cormack (1970) claimed that cells become less antigenic after surface sialic acid on Walker tumor cells was removed by neuraminidase. Currie and Bagshawe, and Cormack both used neuraminidase prepared from the same source-V. cholerae. In the comparative study of immunogenicity, MC tumor cells which were incubated with purified neuraminidase derived from V. cholerae were also able to produce tumor immunity in the present system. The failure in immunoprotection by the cells pretreated with neuraminidase obtained from C. perfringens cultures could be attributed to structural degradation of the membrane of MC tumor cells. It was found that cells treated with this enzyme, lost their viability as assessed by trypan blue dye exclusion. Cytotoxic activities of other contaminant enzymes in commercially avialable neuraminidase including the one we used (C. perfringens), have recently been reported (Kraemer, 1968).

As Sendai virus possesses neuraminidase activity (Sokol et al, 1961), one could argue that the effect of viral neuraminidase might play a major role in anti-tumor immunoprotection in mice treated with virus-fused tumor cells. This possibility could be ruled out in view of the following findings. First, pH of Sendai virus suspension harvested from allantoic fluid of hen eggs usually ranged from 7.6 to 7.8.

After storage in the cold (4°C) for 2 to 3 weeks, the pH of the suspension rose to 8.0 to 8.2. The optimal pH for neuraminidase of egg grown Sendai virus was shown to be 5.0 to 5.5 and very low activity could be found when the pH was at or above 7.0 (Tozawa et al, 1967). In our cell fusion process, pH of virus suspension was always higher than 7.4. The reaction of neuraminidase in virus-fused cell immunogenicity, therefore, appeared to be less likely. Secondly, the results of the pilot and one of the controlled experiments indicate that virus treated single MC tumor cells with various doses did not effectively produce immunoprotection against the tumor growth in such treated animals. Finally, the immunity induced by neuraminidase treated tumor cells declined on repeated challenges while that induced by virus-fused cells did not.

As mentioned earlier, despite the relative stability of chromosome pattern in the MC tumor, variability in transplantable tumor cell population is not unusual (Hauschka, 1952). The occurrence of random fusion between or among tumor cells constituted a mosaic type of immunogens when such fused cells were injected into the mice. This probably is an advantage with regard to wide spectrum of anti-tumor immunity so that no tumor variants could escape from the immunological attack.

It must be kept in mind that the response of the body to virus-fused multinucleate tumor cells suddenly induced by inactivated Sendai virus would be very different from the host reaction to established polyploid tumor cells. It has been

shown that near tetraploid ascites cells derived from the near diploid cells became more nonspecific with respect to host transplantability (Hauschka et al, 1956; Hauschka and Amos, 1957). In the present study, MC tumor cells was chosen as a model to test the host reaction to polykaryocytes which are potentially polyploid cells. In the former case, it has been suggested that diminished antigenicity may occur by increases in cell volume and alterations in geometry associated with increases in ploidy, which may reduce the density of antigenic sites (Hauschka and Amos, 1957).

Foot-pad cutaneous reactions in mice have been used as indices of experimental delayed hypersensitivity (Nelson and Mildenhall, 1967; Wang and Halliday, 1967; Halliday and Webb, 1969). Our work has shown that tumor specific delayed hypersensitivity and the ability to suppress the growth of tumor cells can be demonstrated in the immune mice by the same test. It should be pointed out that delayed reaction and tumor suppression did not always accompany each other unless viable tumor cells for injection were at an appropriate concentration. The number of live cells needed to elicit an effective delayed hypersensitivity reaction was established in our system and shown to be approximately 1×10^5 cells. In addition, various forms of tumor antigen preparations were used for foot-pad injection. Only living cells were found to give a suitable reaction. Wang's observation on delayed hypersensitivity to benzpyrene-induced tumors in rats (Wang, 1968) differed in one aspect from that reported here. She used sonically

vibrated tumor extracts as eliciting antigens and found them effective. In contrast, we found that mechanically disrupted cell materials had no significant activity.

Although the mechanism of antigen transport is not known, regional lymph nodes are important sites of antigen processing and cellular changes occur in these organs characterized by the appearance of large pyroninophil cells (LPC) (Gowans, 1962). Gowans and McGregor (1965) stated that there is good evidence that the precursor of LPC is the small lymphocyte which transports antigens to the lymph nodes. They also considered that the activated lymphocytes released from lymph nodes could conceivably be the progeny of LPC. In another connection, Weir (1967) has recently suggested that the form which an antigen exhibits may determine the response. Particulate antigenic material phagocytosed by primary antigen handling cells (macrophages) appear to stimulate immunity, while soluble cell constituents which contact lymphocytes directly induce tolerance. For the former, the antigen is bound non-specifically to macrophage RNA (Fishman and Adler, 1967) and transported to a lymphoid cell forming antigen-RNA complex and proliferation of lymphoid cells initiated. Intimate contact between lymphocyte and macrophage have been observed and antigenic information may be transferred (MacFarland and Heilman, 1965). The concept of lymphocytes with changing morphology and function seems to fit well with current understanding that the potentially long-lived, small lymphocyte is not an end cell. The alternative pathway leads

to the formation of plasmocytes which engage in immunoglobulin production. Clues to the mechanisms of rejection have been derived from *in vitro* studies, though they may not truly be parallel to rejection in the body. Experiments *in vitro* using sensitized lymphoid cells for cytolysing the target cells, all seem to require a close contact between immune lymphoid cells and target cells (Rosenau and Moon, 1961; Möller and Möller, 1965) without involvement of complement (Phillips *et al*, 1968). Takasugi and Hildemann (1969 a and b) suggested that the actual immune rejection could involve contact between lymphocytes and target cells, bringing IgM antibody bound or free of lymphocytes into the immediate proximity of the tumor cells.

Alexander (1967) has demonstrated chemically induced tumor regression following injection of immune lymphoid cells. The injected immune lymphoid cells did not reach and exert a direct effect on the tumor cells, but settled mainly in lymphoid organs and predominantly in spleen in which the lymphoid cells' RNA appeared to initiate enhanced host resistance. Recent evidence for "immune RNA" in antibody formation and in the transfer of transplantation immunity supports this view (Sabbadini and Sehon, 1967; Bell and Dray, 1969; Kuecheler and Rich, 1969). A number of experiments with solid tumors indicate immune lymphoid cells (or their RNA) serve to initiate a process which is then completed by the host (Alexander *et al*, 1966). The anti-tumor effective cells are likely the LPC

that begin to release from a regional node in three to four days after injection with immune lymphoid cells. If this is the mechanism of immune cell action in the adoptive transfer of immunity, multiple transfers of immune lymphoid cells might be important for the results of our experiments obtained using immune lymph node cells (Table XII). Transferrence of immune cells to the virgin mice should be similar to a process of immunization. Nevertheless, the action of injected immune cells against ascites tumor cells probably operated on a different basis, since it has been shown that the direct effect of immune lymphoid cells on the ascitic cells resulted in destruction of the latter in vivo (Alexander et al, 1966).

The ability of regional lymph node cells to confer the adoptive transfer of immunity was confirmed by use of graded tumor doses for challenge. The number of splenic lymphoid cells required to effect the adoptive transfer of chemically induced tumor immunity was approximately the same as the number of lymph node cells and the degree of immunity produced was comparable (Bard et al, 1969). In the present experiments, the failure to demonstrate a complete immunoprotection is not well understood. The possible explanation might involve the mechanism of immune cell action just discussed. It might also be due to a high tumor dose (1×10^5 cells) used, as partial protection was indicated by prolonged latent period of tumor appearance and longer survival in the spleen treated animals. Moreover,

animals given a mixture of immune spleen and tumor cells at a higher ratio (100:1) survived significantly longer than the control animals injected with tumor cells alone (Table XI). In this connection, the spleen immune cells in the experiments could confer a detectable degree of immunity in terms of complete tumor resistance, if lower and different graded tumor doses had been tested.

In addition to the enlargement of spleen and lymph nodes, one of the chief characteristics of active immunity is cell proliferation indicated by lymphocytosis (Takasugi and Hildemann, 1969b; Bloom and Hildemann, 1970). The *in vitro* induction of transformation and proliferation by specific antigens might represent an expression of the *in vivo* delayed hypersensitivity reaction. Lymphocytes obtained from individuals who were sensitive to a particular antigen, such as tuberculin, transformed into proliferating immunoblasts when exposed to the same antigen *in vitro* (Pearmain *et al*, 1963; Mills, 1966; Oppenheim, 1968). The *in vitro* proliferation, therefore, may be related to *in vivo* function. Some immunologically committed lymphocytes (potentially long lived cells) continue their immunological role, with rapid transformation into proliferating cells and presumably antibody formation, in the event of re-exposure to the antigen to which the cells have been committed (Gowans, 1962). It has been shown that proliferating blasts *in vitro* are cytotoxic to cells against which they are sensitized (Holm and Perlman,

1965; Möller, 1967). The cytotoxicity demonstrated specificity toward histocompatibility antigens of the cells used in immunization, therefore, establishing the cell-mediated immune response.

In the present experiment on cellular responsiveness of splenic lymphoid cells, a remarkable increase in spontaneous DNA synthesis was found to occur in cultures of spleen cells from immune animals (15 days after challenge) (Table XIII). These *in vitro* results represent an ongoing immune response in the actively immune mice. This finding is in accord with lymphocytosis which is assumed to be intimately involved with the tumor-specific rejection. In view of results obtained from this experiment and from the test of time sequence for delayed hypersensitivity (Fig. 34), onset of rejection must have occurred 4 to 6 days after the challenge and continued for more than 20 days. Spontaneous transformation (or DNA synthesis) *in vitro* has also been used as a possible test for homograft compatibility (Bach and Hirschhorn, 1964) and for drug hypersensitivity (Caron and Sarkany, 1965) based on the absence of spontaneous transformation in normal control culture.

The spleen cells from the immune animals failed to show significant stimulation by PHA at optimal concentration (1:25) when compared with controls. This can be attributed to failure of immune "cytotoxic" lymphoid cells to respond to the non-specific stimulator-PHA (MacLennan and Harding, 1970).

A general decrease of immunological reactivity in tumor-bearing hosts is compatible with the anergy often demonstrated in animals carrying experimental tumors (Stjernswärd, 1965) as well as in human cancer patients (Lamb et al, 1962; Amos et al, 1965; Hattler and Amos, 1965; Robinson and Hochman, 1966). The absence of delayed type of hypersensitivity and lack of resistance to tumor challenge in tumor-bearing mice might have a common cause. It may be a state of immunological commitment in the host induced by the excess tumor antigens or an immunological deficiency associated with the tumor's rapid growth (Smith and Alder, 1970). This has been further explored using in vitro lymphocyte stimulation by PHA in spleen cells from chemically induced tumor bearing mice, and the results were compared with spleen cells' PHA response of animals immunized with other antigens (Adler, 1970). In combination of spleen cell separation and ^{3}H tdr incorporation assay, the same author was able to demonstrate that small lymphocytes (PHA reactive cells) in subpopulation of the spleens of tumor-carrying-mice decreased in their proportional representation in the spleen and total reactivity of the spleen cells to PHA was reduced. The increased subpopulation in the case was found to be antibody producing cells or their precursors. Furthermore, similar changes occurred in the spleen cells of animals immunized with either sheep red blood cells or allogeneic cells. These results suggest that decreased PHA reactivity found in the spleen cell population of tumor-bearing mice may be related to immunological commitment rather than immunological deficiency.

The results of immunofluorescent studies yield a positive correlation of MC tumor with the serum obtained from immune mice, although no attempt was made to quantitate the percentage of immunofluorescent cells. The reaction was specific, as negative results were obtained when SP tumor sections were tested. The observations are similar to those obtained in the studies with 3-methylcholanthrene induced mouse sarcoma (Lejneva et al, 1965). The presence of tumor-specific antigens at the cell periphery was demonstrated in this experiment. The antigens (TSTA's) by their nature must be localized on the cell surface. It seems probable that antigens located deep within the cytoplasm are not effective in eliciting significant antitumor immune response. Applications of this technique have produced evidence of the presence of circulating antibody in the immune mice, since only serum from immune mice gave the positive results. The studies also have shown the site of localization of antigen-antibody complexes, which were found to be in the cell membrane and cytoplasm of the tumor cells.

Humoral antibody in the immuneserum was also demonstrated by its effect on the enhanced growth of treated MC tumor cells in the syngeneic recipients (Table X). The degree of enhancement is comparable to that previously reported against methylcholanthrene induced sarcomata (Möller, 1964; Koldovsky, 1969). However, a low dose of immune serum did not inhibit growth of transplanted tumor, as observed by Koldovsky (1969). At present, we have no satisfactory explanation for the difference in results.

Studies of the absorption of agglutinating antisera indicate that sera from immune animals contained higher titers of tumor agglutinin. The significance of agglutination and its exact role in anti-tumor immunity is difficult to assess. However, it suggests a strong affinity between antigen and antibody at the antigenic sites of tumor surface, leading to immunological enhancement. Moreover, it appears that there is no direct relationship between the titers of agglutinin in sera and the degree of spontaneous DNA sythesis in vitro of splenic lymphoid cells in the immune mice.

Some non-viral fusion agents, namely, lysolecithin, retinol and sodium nitrate have recently been described (Poole et al, 1970; Lucy, 1970; Power et al, 1970). However, these agents appear to be toxic to the cells, although they easily produce fusion in some types of cells. If cytotoxicity of these substances could be reduced, it is feasible and would be important to use such fusion agents to investigate the actual mechanisms underlying immune reactions in fused-cell exposed animals. In such a system, viral antigenicity and viral induced interferon could completely be eliminated from the tumor-host interaction. Furthermore, further studies using more refined cell separation techniques and various routes for immunization in animals are required to help our understanding of the role of fused cells in anti-tumor immunogenicity. It is hoped that research along this line will lead to successful approaches to cancer immunotherapy.

SUMMARY

A transplantable methylcholanthrene induced mouse tumor was examined by light and electron microscopy. Light microscopic observations revealed that the tumor was a sarcoma and made up of undifferentiated anaplastic cells exhibiting cellular and nuclear pleomorphisms. Examination of the tumor ultrathin sections showed that the cytoplasm of most tumor cells were filled with ribosomes. The overall appearance of the tumor by electron microscopic obervations suggested that they were composed of one general cell type. The tumor cells were characterized by stemline karyotypes of 43 chromosomes, including always one or infrequently two metacentric marker chromosomes.

By using UV-inactivated Sendai virus-induced-cell fusion technique, a model system of anti-tumor immunogenicity has been developed, based on the loss of transplantability of the fused tumor cells and resistance to subsequent tumor challenge in the isologous hosts, A/Jax mice. The nontransplantable fused tumor cells serving as immunizing doses proved highly immunoprotective in animals, as they induced heightened resistance to subsequent viable tumor challenges. The immunological rejection involved in this resistance was tumor-specific. Sendai virus treated single tumor cells of various doses failed to protect the animals from tumor growth. Furthermore, the active immunity against the tumor induced by the present method was compared

with those of other immunizing techniques, namely, by using γ-irradiated tumor cells and neuraminidase treated tumor cells. The virus fused cell treatment induced a better protection than the other two procedures in the animals tested. Three repeated injections of fused cells at 10 day interval conferred a higher level of immunity against tumor challenge.

The establishment of the state of active immunity in fused-cell immunized mice was further supported by the test of delayed hypersensitivity, using an appropriate viable tumor inoculum (1×10^5 cells). Tumor cells injected into the foot-pads of the actively immune mice gave positive reactions. The live tumor cells used in the test were always rejected. The adoptive transfer of tumor immunity from immune mice to the normal syngeneic mice was also accomplished by the intraperitoneal administration of cells from lymph nodes.

To test cellular response of lymphoid cells from tumor immune mice, the proliferating capacity of spleen lymphoid cells was assessed by tritiated thymidine incorporation in vitro. A high degree of spontaneous DNA synthesis of immune spleen lymphoid cells was evident, as a 23-fold increase of the radioactive counts was obtained when compared with controls. This indicates an ongoing immune response occurred in the immune animals which had been challenged 15 days before the tests. A similar suggestion could also be reflected by the results of relatively poor net-response of immune spleen cells to further non-specific stimulation by PHA in culture.

Humoral antibody against MC tumor specific antigens in

immune animals was demonstrated *in vitro* by application of the indirect immunofluorescent antibody technique. Immune humoral antibody was also demonstrated *in vivo* by its effect on enhanced growth of MC tumor cells after the serum incubated cells were transplanted into syngeneic recipients.

Finally, the possible mechanisms of anti-tumor immunity revealed from these results were considered in relation to pertinent literature of tumor immunology.

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