A STUDY OF TUMOR IMMUNITY IN THE GUINEA PIG

by

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Statement of the Problem

The major paradox confronting tumor immunology today is how tumor cells escape cell-mediated immune destruction in hosts with demonstrable tumor immunity. One widely-held explanation to account for this paradox views a competitive relationship between cell-mediated anti-tumor immunity and specific humoral enhancing factors (antigens, antibodies, immune complexes?), favoring tumor cell survival and growth. Other explanations to account for the escape of tumor cells from immune destruction, however, should also be considered. Recently, non-specific humoral factors have been implicated in suppression of cell-mediated immunity in a number of non-tumor systems. It is conceivable, therefore, that these factors may also compromise cell-mediated tumor immunity and thereby encourage development of malignancy. Unfortunately, animal models in which to study these non-specific humoral factors are unsuitable or unavailable.

The purpose of this research project, therefore, is a) to develop a suitable animal model system to study tumor immunity and b) to investigate the influence of non-specific humoral factors on tumor growth, as a possible mechanism to account for escape of tumor cells from immune destruction.

Initial experiments describe the development of the model and include a) the carcinogenic induction of tumors in the guinea pig and b) a description of the model in terms of growth rate, histology, malignancy, development of immunity and tumor specificity. In additional experiments, *in vivo* and *in vitro* assays for detecting host-protective tumor immunity are evaluated. Following development of the

tumor model, experiments are designed to determine the role of non-specific humoral factors on progressive tumor growth, i.e., factors which may nonspecifically aid in the escape of tumor cells from lymphocyte-mediated destruction. Included in this section are experiments describing the detection, assay and specificity of these factors, as well as their relationship to immunosuppression and tumor growth.

Historical development of tumor immunology

An immunological approach to cancer therapy gained recognition when Gross (1), in 1943, demonstrated that host-protective tumor immunity could be stimulated in inbred animals. In this study, Gross produced immunity to chemically-induced (3-methylcholanthrene, MCA) sarcomas in inbred C3H mice by intradermal inoculation of viable tumor tissue. He proposed that this immunity was directed specifically against antigens arising on the tumor during malignant transformation, and that the new antigens might have immunotherapeutic value. This work met with skepticism, however, because mutations occurring in the tumor during repeated passage or minor immunogenetic differences in the C3H mouse strain were considered as possible explanations for the observed results (2,3). In 1953, Foley (4) offered convincing support of Gross' observations using both spontaneous mammary carcinomas and chemically-induced sarcomas in the same C3H mouse strain. Each tumor type, used early after induction, stimulated tumor-specific immunity following tumor ligation. Foley reasoned that since the same C3H mouse strain was used in all experiments, the specificity of the tumor immunity must have been due to antigenic differences among the tumors

themselves.

In 1957, Prehn and Main (5) presented the strongest evidence that normal and neoplastic tissue differed antigenically, and that the tumor antigens themselves were responsible for stimulating host-protective tumor immunity. Animals immunized to MCA-induced tumors routinely accepted skin grafts but rejected tumor transplants from the same donor animal. These experiments demonstrated conclusively that certain neoplasms expressed unique tumor-specific antigens (TSA) which stimulated tumor immunity. Klein et al (6) subsequently confirmed these studies.

The question of whether or not unique tumor antigens were expressed on viral-induced tumors was considered by a number of investigators in the early 1960's. Viral-induced tumors of either DNA (7,8) or RNA (9,10) virus origin were characteristically rejected in tumor-immunized, syngeneic hosts. It is now apparent that a large number of viral-induced, chemically-induced, and certain spontaneously-derived tumors, if not all types of tumors, differ antigenically from the tissue of their origin (11-21).

Previous studies also pointed out that antigenicity and specificity vary widely in the tumor system examined. In general, the available evidence indicates that immunity to chemically-induced tumors has unique specificity, but that tumors induced by a given virus have common specificity (7-21). In this regard, immunity to a viral-induced tumor protects the host from other tumors induced by the same virus, but immunity to a chemically-induced tumor confers little or no protection to other tumors induced by the same carcinogen.

Although tumor immunity could be readily produced, it was not clear whether host protection was conferred by the humoral component of the immune response (antibodies), the cell-mediated component (immune lymphocytes), or both. Investigations by Mitchison (22), in 1954, Klein and Sjögren (23), in 1960, and Old et al (24), in 1962, were designed to answer this question. By transferring either serum antibodies or immune cells from tumor-immunized to normal animals, and subsequently challenging the recipients with viable tumor, they were able to distinguish between antibody-mediated and cell-mediated immune protection. Using the passive transfer technique, they concluded that both cell-mediated and humoral immunity were produced in response to injections of viable tumor tissue, but that the cell-mediated component was responsible for host-protection from the tumor. Klein et al (6) confirmed the importance of cell-mediated immunity for host protection by demonstrating inhibition of tumor growth in mixtures of tumor cells and immune lymph node cells transferred to isogeneic irradiated mice. Klein and Sjögren (23) transferred tumor protection to normal recipient mice with similar mixtures of cells. Reports by other investigators added convincing evidence that cell-mediated immunity was indeed responsible for host protection from tumor growth (25-27).

During this same period, studies by the Hellströms and co-workers (28,29) and Good et al (30) revealed that the humoral immune response was not effective in preventing tumor growth. In addition, the Hellströms and other investigators proposed that tumor-induced antibody formation might even account for enhanced tumor growth, observed when serum from tumor immune animals was passively transferred to recipient

animals bearing tumor transplants (29,31). In this respect, it was postulated that tumor enhancing antibodies might prevent cell-mediated destruction of tumor cells (25,32). A number of reports have since shown that anti-tumor antibodies can inhibit the cytotoxic effects of cell-mediated immunity in both experimental (15,23) and human (26,27, 29,31,33) malignancies. In addition, in vitro experiments have shown that anti-tumor antibodies might block the cytotoxic activity of immune lymphocytes and inhibit their ability to prevent tumor colony formation (26,28,33-36).

The above experiments imply that the paradox of progressive tumor growth in the face of cell-mediated tumor immunity may be partially explained by blocking or enhancing antibodies. Other specific or non-specific humoral factors may also play a role in the inhibition of cell-mediated tumor immunity.

In 1973, a report by Alexander and Currie (37) indicated that specific humoral factors, such as immune complexes or circulating tumor antigens, might also enhance tumor growth. Enhancing antibodies or immune complexes could directly mask tumor cells from lymphocyte-mediated destruction by covering antigenic sites required by lymphocytes for immune recognition. Alternatively, immune complexes or soluble tumor antigen could prevent tumor destruction by competing with tumor cells for specific antigen receptors on sensitized lymphocytes.

The contribution of specific humoral factors (antibody, tumor antigen, or immune complexes) in allowing the escape of tumor cells from cell-mediated immunity is currently being pursued in many laboratories. Investigations within the last fifteen years have also

demonstrated that non-specific factors might be involved in suppression of immunity, and therefore in the escape of tumor cells from immune destruction. Although the majority of reports have been in non-tumor systems, the potential importance of these non-specific factors in suppression of cell-mediated responses to tumor antigens should be considered. The involvement of non-specific suppressive factors in the escape of tumor cells from immune destruction has not been critically evaluated because of the lack of a suitable animal model. It is, therefore, the dual purpose of this research project to: a) develop a suitable animal-tumor system which can serve as a model for future studies, and b) investigate the influence of non-specific humoral factors on tumor growth and tumor-associated immunosuppression as a possible mechanism to account for escape of tumor cells from immune destruction.

Animal models for studying tumor immunity

The major paradox confronting tumor immunology today is how tumor cells survive and replicate, despite an apparently active and functional cell-mediated immune response of the host against the tumor. To resolve this question, an animal-tumor model is needed in which cell-mediated immunity can be readily produced, detected and assayed. Some of the problems associated with current tumor-animal models and reasons why these models are not well-suited or adaptable for this kind of study will be considered below.

Until the early 1960's, most investigations in tumor immunology used the inbred mouse as an animal model. A tremendous amount of

information was gained by studying the immune response of this species to tumors induced by chemical or viral carcinogens, or to tumors of spontaneous origin. In 1960, however, when Klein et al (6) demonstrated that anti-tumor immunity was mediated by immune cells, rather than humoral antibodies, a need for other animal models in which detection and quantitation of cellular immunity was easier than in the mouse became apparent. Other inbred species that were available included chickens, rats and guinea pigs. Cell-mediated immunity, however, was as equally difficult to detect and quantitate in the chicken and rat as in the mouse. In addition, spontaneous tumor development was common in some of the strains of these species.

Within the last several years, the inbred guinea pig has been considered an excellent choice for a model of this kind (38). Cell-mediated immunity in this species has been well-defined and assays of cellular immunity have been described both in vivo and in vitro. Two inbred strains of guinea pigs, Strains 2 and 13, are available. Although guinea pigs are relatively expensive to feed and house when compared to mice, they are fairly small and convenient to work with in the laboratory. In addition, passive transfer of cell-mediated immunity has been demonstrated in the guinea pig, using both immune cells or cell-free extracts (transfer factor, TF). This has not been possible to date in other inbred species. Furthermore, spontaneous tumor development has not been reported in the guinea pig.

Two laboratories (39,40) are currently developing the guinea pig as a model to study chemically-induced tumors, but progress has been slow and difficult to evaluate. The tumors under consideration have

not been routinely malignant and, in addition, have been rarely available for study by other investigators. The pertinent literature describing these models will be considered below.

The first tumor transplants in guinea pigs were reported by Jones (41) and Murray (42) in 1916. In this study, they demonstrated temporary growth of sarcomas and carcinomas in outbred recipients. Esmarch (43), in 1942, Shimkin and Mider (44), in 1940, and others (40,45) have since demonstrated long-term growth of chemically-induced sarcomas and hepatomas in inbred guinea pigs. Argus and Hoch-Ligeti (39), in 1963, first reported that the water soluble carcinogen, diethylnitrosamine (DEN), induced hepatomas in nearly 100% of randomized guinea pigs when supplied in the drinking water. In addition, they demonstrated metastases to other organs including lung and lymphoid tissue. These initial results were subsequently confirmed by Crisler and others (46).

The DEN-induced hepatoma has been the most widely examined tumor model in the guinea pig to date. The biological and pathological characteristics of the DEN-induced hepatoma, as well as its antigenicity in Strain 2 guinea pigs, were described by Rapp (47) in 1968. He showed in this study that the tumor was readily transplantable in both its original solid form (Line 1), and as an ascites variant. In addition, Rapp demonstrated that if living Line 1 tumor cells were injected intradermally into normal syngeneic guinea pigs, a small tumor papule formed, which eventually ulcerated and regressed. Animals treated in this manner were protected from subsequent tumor challenge by intradermal or intramuscular routes. Rapp also demonstrated in this same report that tumor immunity could be evaluated in immunized animals by

intradermal skin test reactivity to viable tumor tissue. Churchill (48), employing the same tumor system, reported in 1968 that delayed skin reactions to DEN-induced tumor tissue were the same as delayed skin reactions noted in other antigen systems, as judged by rate of development, onset, duration, histology and specificity.

Similar findings were reported by Gross (1,49), in 1943, who observed that intradermal inoculation of a methylcholanthrene-induced tumor was an effective method of producing immunity in inbred mice. In addition, Gross (49) and others (50,51) demonstrated that this route of inoculation was more effective than any other in producing tumor immunity. These results suggested that stimulation of tumor immunity depended, at least partially, on the route of inoculation. The mechanism for development of immunity following intradermal inoculation of tumor tissue, however, remains obscure.

Kronman (38), in 1970, demonstrated that the DEN-induced tumor system might serve as a useful model to study tumor immunotherapy in the guinea pig. He showed that if weekly intradermal injections of viable tumor were given within 5 days after an intramuscular tumor challenge, systemic immunity was produced which prevented or delayed growth of the tumor challenge. Over 30% of the animals treated in such a manner were afforded complete protection. Kronman noted, however, that host-protective therapy in this system depended on the number of cells in the challenge injection, as well as the route of injection.

Wepsic (52), in 1970, demonstrated that tumor cell viability was also an important consideration for successful intradermal immunization of Strain 2 guinea pigs with the DEN-induced hepatoma. He noted that

tumor cells cultured *in vitro* were protective, but those subjected to freeze-thaw cycles or X-irradiation were not effective in stimulating immunity.

Conversely, Eilber (51) reported in a similar guinea pig tumor system that irradiated liposarcoma cells or liposarcoma cells grown in tissue culture were somewhat effective in stimulating immunity. Although Eilber's results have been supported by similar investigations from a number of other laboratories (53), it is unclear whether tumor cell viability is a requirement for stimulating immunity.

More recently, studies in the guinea pig have demonstrated that chemically-induced tumors have both unique and shared tumor antigens (54,55). Using DEN-induced tumor cell lines, Zbar (56), in 1969, observed that antigenic differences could be detected in 5 of 6 tumors by cross-challenging immunized Strain 2 guinea pigs with viable tissue from each tumor type or by delayed cutaneous hypersensitivity. In contrast, however, he noted that antigenic shifts occurred in certain of the tumors examined during *in vivo* passage. Changes in antigenic character during *in vivo* passage were also observed by Takeda (57,58), using a MCA-induced sarcoma system in rats. The mechanism responsible for these antigenic shifts is still unknown; however, such antigenic changes should be considered when evaluating any model used to study tumor immunity.

Detection of tumor immunity with soluble antigen preparations from tumor cells has recently received attention. Early attempts to extract tumor antigens were based on methods designed to liberate transplantation antigens from normal guinea pig tissue (59-61). These procedures

included sonication, homogenization or enzymatic digestion of lung, spleen, kidney or liver tissue.

Kahan et al (62), in 1969, and Holmes et al (63), in 1969, used sonication to release tumor-specific antigens from several different chemically-induced sarcomas in Strain 13 guinea pigs. In both reports, the extracted tumor antigens elicited delayed cutaneous hypersensitivity responses in pre-sensitized allogeneic hosts. Further, Kahan (59) demonstrated that guinea pigs specifically immune to one type of tumor were unable to respond to soluble antigens isolated from other tumor types. In a subsequent report, however, Holmes (63) found that 2 of 3 different syngeneic methylcholanthrene-induced sarcoma lines elicited cross-reacting delayed skin test responses, and therefore shared tumor antigens. Studies concerning antigen specificity of these sonicated tumor preparations are still being conducted.

More recently, Meltzer (64), in 1971, isolated soluble tumor-specific antigens from DEN-induced guinea pig hepatomas by hypertonic salt extraction with 3 molar potassium chloride (KCl). This method, originally used to isolate human histocompatability antigens from leukocytes (65,66), resulted in a high recovery of immunologically specific tumor antigens. Under optimal conditions, Meltzer reported a recovery of 15-40% of the original antigenic activity of whole, viable tumor cells, as measured by delayed skin reactivity in immunized animals. A subsequent report by Meltzer (67), in 1972, demonstrated that soluble tumor antigen activity in the guinea pig could also be measured in vitro by macrophage migration inhibition or lymphocyte transformation.

Recent reports on the DEN-induced tumor in the guinea pig have described its potential as a model to study mechanisms of immunotherapy. In one series of experiments using the Strain 2 guinea pig hepatoma, Zbar (68), in 1970, proposed that prevention of tumor growth by cell-mediated immunity required more than one mechanism. He demonstrated that tumor antigens were initially recognized by host cells in a specific manner but that subsequent tumor cell destruction occurred by a non-specific mechanism. Another study by Zbar (69), in 1970, demonstrated that the rejection of tumor cells was initiated by cell fractions containing lymphocytes and neutrophils (Step 1), but that cell destruction was dependent on a macrophage-rich cell fraction from either immune or non-immune donors (Step 2). Bernstein (70), in 1971, added support to Zbar's proposed second step by injecting tissue culture fluids containing migration inhibition factor into Strain 2 guinea pigs. At the intradermal site of injection, mononuclear cells appeared and induced an inflammatory reaction. Growth of tumor cells subsequently injected into these sites was inhibited.

Because the second stage of tumor destruction appeared to occur by a non-specific mechanism, attempts were made to evaluate this step as a possible approach for immunotherapy. A report by Old (71), in 1961, demonstrated that the Bacillus of Calmette-Guérin (BCG) provoked a non-specific response of the reticulo-endothelial system (RES) in mice, leading to inhibition of growth of a transplanted sarcoma. In expanding these studies in the guinea pig, Zbar et al (72), in 1971, noted that growth of syngeneic guinea pig hepatomas was inhibited if the tumor cells were mixed with BCG before intradermal inoculation. Zbar observed

similar results when BCG was injected directly into the tumor site (73). Guinea pigs treated in this manner developed specific delayed cutaneous hypersensitivity to the tumor tissue (74). Tumor growth, however, was suppressed only if BCG preparations were viable or if cell walls from BCG were attached to oil droplets (75). Mathé (76) reported, in 1969, that human patients with acute lymphoblastic leukemia also appear to respond to BCG treatment by exhibiting at least partial or temporary remission of their disease.

It is clear, however, that further evaluation of this potential method of tumor immunotherapy is critical for both an understanding of the process itself and to determine its possible use as a non-specific promoter of the immune response. If the two-step mechanism of tumor destruction proves to be correct, specific tumor enhancing factors (antibody, antigen, immune complexes) may be found to block the recognition stage (Step 1), and non-specific inhibitors of cellular immunity may be found to block the tumor destruction stage (Step 2).

In summary, the guinea pig has been used as a model for cancer immunotherapy by two laboratories in this country. Although valuable information has been derived from these studies, the unavailability of this model and the inconsistently malignant nature of the DEN-induced hepatoma have limited its usefulness to studies evaluating effectiveness of immunotherapy. Furthermore, Strain 2 guinea pigs are no longer commercially available for study. This project was designed, therefore, to develop a tumor model in Strain 13 guinea pigs with a malignant and lethal tumor that stimulates detectable cell-mediated immunity. With this type of model, experiments can then be designed to investigate the

paradox of how tumor cells survive in animals demonstrating tumor-specific cell-mediated immunity.

Non-specific suppression of the immune response

Non-specific humoral factors have recently been shown to suppress both cell-mediated and humoral immunity to a number of antigens. Consequently, these factors may also suppress tumor immunity and thereby allow tumor cells to escape lymphocyte-mediated tumor destruction. The mechanism of suppression, although unclear, may involve non-specific inhibition of the tumor-destruction stage of tumor immunity proposed by Zbar (69). With the use of the guinea pig as a suitable animal model, the objective of this project is to determine if non-specific suppressive factors can be detected in tumor-bearing hosts, if they express a relationship to tumor growth, and if they have the potential to suppress cell-mediated tumor immunity and thereby account for the escape of tumor cells from immune destruction.

A review of the relevant literature describing the discovery, isolation and partial characterization of non-specific humoral factors which could play a role in suppression of tumor immunity will be discussed below. The mode of action of these factors and the major systems in which their activity was observed will also be described.

Kamrin (77), in 1959, was the first investigator to demonstrate that large doses of crude alpha globulin fractions from normal plasma could significantly suppress homograft rejection. Both rat and human alpha globulins, isolated by a cold ethanol precipitation procedure, were effective. Mowbray (78), in 1963, subsequently confirmed these

studies using alpha globulin fractions isolated from serum by chromatographic methods. In this report, skin allograft survival was significantly prolonged in rabbits and rats using alpha globulins from bovine, rat, rabbit and human sources. Another report by Mowbray (79), in 1963, described the immunosuppressive effect of alpha globulins from bovine sources on humoral immunity. He observed that the production of antibodies in rabbits to human serum albumin or human red blood cells was diminished when alpha globulins were present during the inductive phase of antibody production.

Failure by other investigators to repeat Mowbray's experiments, however, led to skepticism with respect to the immunosuppressive nature of alpha globulins (80,81). As a result, interest in these suppressive factors declined. Mannick and Schmid (82), however, in 1967, and Bondevik et al (83), in 1968, isolated an immunosuppressive alpha globulin fraction from normal human plasma which prolonged skin allograft survival in both rabbits and mice, following a single intravenous injection of the active fraction. They also noted that the active fraction, an "immunoregulatory alpha globulin" (IRA) fraction, differed from Mowbray's active fraction in two important ways. First, IRA was completely nontoxic to bone marrow, spleen and lymph node tissue, and to isolated lymphocytes from rabbits (83). In addition, IRA had no significant effect on hematocrit values, absolute leukocyte or lymphocyte counts and did not impair renal function (82). Second, the IRA fraction did not contain ribonuclease activity, which was present in Mowbray's active fraction. In addition, ribonuclease was reported to have considerable immunosuppressive activity both in vivo and in vitro (84-86).

Further, Mowbray and Scholand (87) demonstrated that pancreatic ribonuclease, in aggregate form or coupled to other proteins, produced similar effects to those of Mowbray's alpha globulin fraction.

In vitro studies initiated by Cooperband et al (88), in 1968, then demonstrated that IRA suppressed lymphocyte transformation induced by either phytohemagglutinin (PHA) or specific antigens in mice. Similar studies in man by Riggio et al (89), in 1969, indicated lymphocyte reactivity was suppressed when serum containing elevated alpha globulin levels was present. Other observations in vitro demonstrated that both primary and secondary immune responses to specific antigens were suppressed by IRA in animals and man (90-95).

Cooperband et al (95), in 1972, suggested that IRA acts as a non-competitive antagonist of lymphoid cells during antigen recognition. Reports concerning the mechanism of action are unclear, but have been interpreted as indicating that IRA specifically inhibits thymus-dependent lymphoid cell activation by interfering with metabolic events soon after contact with antigen (96). It has been proposed by several investigators that the active moiety is an immunoregulatory peptide of low molecular weight which is non-covalently bound to proteins with an electrophoretic mobility in the alpha globulin region of serum (97,98).

Increases in autologous serum alpha globulin levels have also been observed during periods of immunosuppression in man. Riggio et al (99), in 1968, noted elevated alpha globulin levels in the serum of renal transplant recipients undergoing graft rejection and assessed their suppressive activity in vitro. His results indicated that the difference between alpha globulins in the serum of patients undergoing

renal graft rejection and those from normal serum may only be quantitative. In this respect, Riggio proposed that elevated alpha globulin levels might serve as "immune regulators or moderators" to prevent "unnecessary hyperimmunization" during homograft reactions. Further, he hypothesized that increased alpha globulin levels might be responsible for the tolerant or anergic states recognized in certain malignant and autoimmune diseases.

In support of Riggio's observations, alpha globulin changes have recently been associated with malignancy in both animals and man. Glasgow et al (100), in 1972, demonstrated in vivo that IRA impaired cell-mediated immune responses to chemically-induced fibrosarcomas in both non-sensitized and immune mice. Similarly, Ashikawa et al (101), in 1971, noted increased serum alpha globulin levels during the development of malignancy in man and animals. They found that serum from tumor-bearing hosts could prolong skin graft survival and suppress graft versus host reactions. These workers proposed that alpha globulins coat immune lymphocytes and thereby lead to inhibition of immunity at the level of antigen recognition. Most recently, Hinrichs et al (102), in 1973, demonstrated similar elevations of alpha globulin levels during the progressive development of malignant melanoma in hamsters.

Non-specific humoral factors may, therefore, lead to suppression of tumor immunity in both animals and man. The presence of these factors in tumor-bearing hosts may partially explain how tumor cells escape and survive lymphocyte-mediated destruction. With the availability of the guinea pig tumor model, the potential importance of these factors in the development of malignancy was investigated.

MATERIALS AND METHODS

Animals: Inbred Strain 13 guinea pigs (H. R. Rosecrans, Hamilton, Montana and Life Systems, Portland, Oregon) weighing 350-600 g were used in most experiments. Selected animals were periodically checked for histocompatability by skin grafting. All animals were fed guinea pig chow (Purina) ad libitum, with the addition of kale twice weekly and Vitamin C in the drinking water as nutritional supplements. Outbred Hartley guinea pigs (H. R. Rosecrans, Hamilton, Montana) weighing 350-600 g were also used in selected experiments. Outbred 6 week old female Swiss-Webster mice (Simonsen Laboratories, Gilroy, California), used in selected experiments, were housed and fed according to standard procedures. All animals were maintained in accordance with AAALAC (103).

Tumor Induction: Three groups of 12 guinea pigs were injected subcutaneously in the abdominal region with a single dose of 20 mg of 7,12-dimethylbenz[a]anthracene (DMBA), 3,4-benzpyrene (BP), or 3-methylcholanthrene (MCA) dissolved in benzene. Animals in all three groups were examined weekly for evidence of tumor growth. Tumor onset and development were recorded for a duration of 24 months. An established 3-methylcholanthrene-induced tumor line (CMCA), used to determine specificity of tumor immunity, was induced by subcutaneous injection of 5.0 mg of the carcinogen in the abdominal region. The latent period of induction of this tumor, a fibrosarcoma, was 14 months (54, 104).

Tumor Passage:

In vivo - The first palpable tumor to arise from each of the above three groups was transferred to syngeneic recipients after reaching a size of 3-5 cm (average of two perpendicular diameters). Tumor-bearing animals were sacrificed by cervical dislocation, the tumor removed, and minced into 1-2 mm pieces (5-10 mg). Several pieces of minced tissue were transferred subcutaneously (SQ) and bilaterally into the rear flank region of adult Strain 13 guinea pigs with a trocar piece. Later passages were performed by injecting a single cell suspension (SCS) of viable tumor cells. This suspension was prepared by expressing finely minced tumor tissue through a stainless steel wire screen (#20 mesh, Small Parts, Inc.) followed by differential centrifugation. The inocula from different tumor lines or tumor passages were always standardized to contain 1×10^8 tumor cells/ml (Model F Coulter Counter, Coulter Electronics, Inc.). Viability of these suspensions, determined by exclusion of 0.4% trypan blue dye (Gibco) was consistently 85-95%. Tumors selected for serial passage were transferred after growing to a diameter of 5-6 cm. Tumor tissue from the first and selected subsequent passages was stored at -60 C in tissue culture medium (MEM, Gibco) containing 1% antibiotic-antimycotic solution and 20% fetal calf serum (FCS, Gibco) and 10% dimethyl sulfoxide (DMSO).

In vitro - Short-term cultures were initiated by explanting 1-2 mm pieces of tumor tissue. Twenty to thirty tumor explants were incubated at 37 C in 250 ml plastic culture flasks (Falcon Plastics) with 15-25 ml of medium. Two types of media were employed: Weymouths -

containing 20-30% heat-inactivated (30 minutes at 56 C) FCS, 1% antibiotics, 1% L-glutamine, 1% sodium pyruvate, and 1% non-essential amino acids (Gibco) and Eagles MEM - containing 20% heat-inactivated FCS, and 1% antibiotic solution (Gibco). After 48 hours, the explants were removed and incubation continued until monolayers were formed. The medium was replaced every 4 days. Monolayer cultures were transferred by reseeding subcultures with a single cell suspension in a ratio of one to three, following exposure to trypsin (0.025% for 10 minutes at 37 C). Tumor cells cultured in vitro were either stored at -60 C in MEM with 10% DMSO or used as indicated.

Tumor Characterization: The first tumors to arise from each carcinogen-treated group were characterized with respect to in vivo growth rate, gross and microscopic histopathology, and tumor specificity. These criteria were also used to characterize later passages of the MCA-induced tumor line (MCA-1). The DMBA- and BP-induced tumors were stored at -60 C because of their relatively slow growth rates during initial passage, and used only in specificity studies.

Growth rates - Growth rates were determined by measuring tumor size (average of 2 perpendicular diameters) at 4-day intervals after tumor inoculation, until the animals died. The tumor inoculum was injected with a trocar piece or as a single cell suspension as indicated.

Histology - Gross and microscopic histological characterization were performed on samples of tissue collected at the time of tumor passage. Selected tissues were preserved and fixed in 10% buffered

formalin until examined. Color, texture and consistency were noted for each sample prior to microscopic characterization. Tissue slices were then stained with hematoxalin (5 minutes) and eosin (30 seconds) according to standard procedures (105). The degree of mitotic division, cell size and shape, presence of nucleoli, and amount of granule formation were evaluated.

Tumor specificity - MCA-, BP- and DMBA-derived tumor tissues were evaluated with respect to tumor specificity. In initial experiments, animals immune to MCA-, BP-, or DMBA-derived tumor tissues were re-challenged subcutaneously with tissue representing each tumor type. Proliferation of one tumor-type in animals immunized to a different tumor-type was considered evidence for distinct tumor specificity. Conversely, absence of proliferation of one tumor-type in animals immunized to a different tumor-type was considered evidence for shared tumor specificity. The injection sites were examined at 4-day intervals for evidence of tumor growth for a period of 3 months. In other experiments, tumor specificity of MCA-1 was assessed with respect to another MCA-induced tumor cell line, CMCA, derived in a similar manner (54). Animals immune to MCA-1 or CMCA were challenged subcutaneously with a single cell suspension of MCA-1 tissue in the left rear flank and CMCA in the right rear flank. These sites were also palpated at 4-day intervals for 3 months for evidence of tumor growth.

Sensitization to Antigens:

Tumor cells - Normal Strain 13 guinea pigs were injected subcutaneously (SQ), intradermally (ID), or intraperitoneally (IP) with

MCA-1 to stimulate the development of immunity. The inocula consisted of 50% saline suspensions of 1-2 mm tumor pieces, or tumor tissue in the form of homogenates, fine minces or SCS. Constant amounts of tumor tissue were given to each animal by the various routes to facilitate comparision. The animals were examined at 2-day intervals by palpation of the injection site and at areas remote from the injection site for evidence of primary and metastatic tumor growth. Survivors of the initial immunizing injection were rechallenged subcutaneously 16 weeks later with SCS (1×10^8 cells) to confirm immunity. Normal control animals were also injected with SCS to insure that the challenge dose was 100% successful in tumor production. Animals resisting a second SQ rechallenge with SCS were considered immune. Immunity was produced against the CMCA tumor following intradermal injection of a single cell suspension of tumor cells (1×10^8 cells) (106). Stimulation of immunity against the DMBA and BP tumors was produced by SQ injection of a 50% saline suspension of minced tumor tissue, using 1.0 ml per side.

Tumor antigens - Strain 13 guinea pigs were injected SQ in the nape of the neck (9 sites, 0.2 ml site) and in the front footpads (0.1 ml per site) with a total of 20 mg of MCA-1 extracted tumor antigens (TSA, see below). The 2.0 ml inoculum consisted of an equal volume of emulsified TSA and either Freunds complete (FCA) or incomplete (FIA) adjuvant (Difco). A booster injection of 5.0 ml of TSA (4 mg/ml) was given intraperitoneally six weeks later. One week following the booster injection, all animals were bled, skin tested with TSA and challenged with viable tumor tissue to assess the

development of immunity.

Dinitrofluorobenzene (DNFB) - Hartley guinea pigs were sensitized to DNFB (Eastman Organic Chemicals) by topical application (5-6 drops) of 2% (v/v) DNFB in absolute ethanol for six consecutive days. The solution was applied in a 2 cm^2 area in the nape of the neck after clipping hair around the site. Control animals were treated with ethanol alone or were untreated. All animals were skin tested five days later with a topical application of 0.5% (v/v) DNFB in olive oil. The reactions were graded 48 hours later according to the following scheme: 0, no detectable reaction; +, patchy erythema; ++, homogenous erythema; +++, marked erythema and raised reaction site.

Tuberculin - Hartley guinea pigs were sensitized to tuberculin by subcutaneous inoculation of 1.0 mg heat-killed Mycobacterium tuberculosis H37RA in 1.0 ml of Freunds incomplete adjuvant, in the nape of the neck. Control animals were injected with incomplete Freunds adjuvant alone. All animals were skin tested intradermally with 10 µg PPD (Parke-Davis) 24 days after inoculation. The reactions were graded by measuring the diameter of induration 24 and 48 hours after skin testing.

Skin grafts - Strain 13 or Hartley guinea pigs were sensitized to allogeneic or syngeneic skin by grafting 1 cm^2 full thickness, abdominal skin bilaterally on the back. The skin grafts were secured and protected with wrap-around bandages (Blenderm, 3M Co.). Normal animals received autologous skin grafts as a negative control for sensitization and to observe the effects of surgery on graft rejection. The grafts were observed at two-day intervals for the first 6 days and

daily thereafter. Rejection was recorded when the grafts became thick, hard and necrotic. Bandages were removed 8 days after transplantation.

Extraction and Isolation of Tumor Antigens: Tumor antigens were extracted and isolated according to the procedure of Meltzer (64) (Fig. 1). One cm^3 pieces of tumor tissue were lyophilized, pulverized, resuspended in 3 molar KCl (14 gm dry wt/150 ml) and stirred for 20 hours at 4 C. The supernatant fluid was collected by centrifuging the mixture at 40,000 g for 1 hour and was extensively dialyzed in distilled water. The water-insoluble material was removed by centrifugation at 40,000 g for 15 minutes. The supernatant fluid was concentrated under a vacuum (Prodicon, Bio-Molecular Dynamics) and returned to isotonicity by dialysis against 0.9% saline.

Assays of Immune Reactivity:

Skin test - MCA-1 and CMCA extracted tumor antigens (20 mg protein/ml) were injected intradermally (0.1 ml per site) in Strain 13 guinea pigs immune to either MCA-1 or CMCA to determine antigenicity and specificity. The degree of induration and erythema was observed after 30 minutes, 6 hours, 24 hours and 48 hours and recorded as the diameter (mm) of the indurated site.

Tumor challenge - Animals immunized to MCA-1 or CMCA by various procedures were rechallenged with viable SCS (1×10^8 cells/ml) prepared from each of the two tumor lines. Each animal was injected with both MCA-1 and CMCA (1.0 ml of each tumor-type per animal) to deter-

mine the extent of immunity and the degree of specificity. The animals were examined at 4-day intervals to determine tumor growth and tumor size.

Migration inhibition - Tumor antigens extracted by KCl were used in the macrophage migration inhibition (MMI) assay to test for immunity to MCA-1 (107). Control and immune animals were injected with 15.0 ml of light mineral oil (viscosity 105, Great Western Chemical Co.) IP, to stimulate peritoneal exudative cells (PEC) (108). Seventy-two hours later, the animals were sacrificed by cervical dislocation and the PEC's collected in 100 ml of Hanks' balanced salt solution (HBSS, Gibco, Inc.). After a single washing (260 g for 20 minutes) with HBSS to remove oil, the cells were resuspended in an equal volume of RPMI-1640 medium (Gibco) containing 5% normal Strain 13 guinea pig serum and 1% antibiotic-antimycotic solution (Gibco) and added to capillary tubes (1.1 - 1.2 mm inside diameter, Sherwood Medical Industries, Inc.). The capillaries were centrifuged at 70 g for 5 minutes, broken at the cell-liquid interface, and added to Sikes-Moore chambers containing 1.0 ml medium with or without TSA (10% v/v, 2.0 mg). The chambers were incubated at 37 C and the cells observed at 24 and 48 hours for migration. Migration was measured by planimetric integration of projected images of the capillaries. Inhibition was recorded if the chambers with antigen migrated less than 80% of controls.

Lymphocyte transformation - Lymphocyte transformation was measured by ^{3}H-thymidine uptake in a modified whole blood culture system (109). Three ml of heparinized blood (60 µ/ml), collected by

cardiac puncture of lightly etherized animals, was diluted to 100 ml with medium RPMI-1640 containing antibiotics and 25 mM Hepes buffer. With the guinea pig, this dilution supplied approximately 4-5 x 10^5 lymphocytes per tube. Where indicated, 5.0 µg phytohemagglutinin (PHA-P, Difco), 0.1 ml of 1/20 pokeweek mitogen (PWM, Difco), or 0.1 ml of concentrations of TSA (0.002 to 200 mg protein/ml) were added to triplicate cultures containing 3.0 ml of the mixture per tube. The cultures were incubated 6 days at 37 C with 1.0 µCi ^{3}H-thymidine (New England Nuclear, specific activity 6.8 Ci/mmole) added 24 hours prior to the end of the incubation period. The cultures were harvested on 2.4 cm glass fiber filters by two washes with 0.15 M saline followed by 3% acetic acid, 5% trichloroacetic acid, and methanol. The filters were placed in 20 ml vials with 10 ml scintillation cocktail (5.0 g 2,5 diphenyloxazole and 0.2 g 1,4-bis-2(4-methyl-5 phenyloxazolyl)-Benzene per liter of toluene, Packard Instrument Co., Inc.) and counted in a liquid scintillation counter (Tri-Carb Model 3375, Packard Instrument Co., Inc.). Control cultures varied less than 10% from the mean. The standard error the mean of triplicate cultures from a single treatment group was calculated according to the following formula:

$$S.E. = \frac{\text{S.D. of triplicate cultures}}{\sqrt{\text{Number of Observations}}}$$

Passive Transfer of Tumor Immunity: Cell-free extracts (transfer factor, TF) prepared from lymphocyte populations of MCA-1 immune animals or normal controls were used to transfer tumor immunity to

normal guinea pigs. Peritoneal exudative and lymph node cells were harvested from each animal following an IP injection of 15 ml of light sterile mineral oil (108,110). The PEC cells were collected in HBSS after three washes of the peritoneal cavity, pooled and centrifuged at 270 g for 20 minutes as above. Suprascapular, axillary and inguinal lymph nodes were excized, minced, and expressed through a #20 mesh stainless steel screen. The peritoneal exudative and lymph node cells from a single animal were then pooled (a small aliquot was removed for testing in the migration inhibition assay), resuspended in 5.0 ml sterile distilled water and subjected to 10 rapid-freeze cycles (-60 C to 37 C) to disrupt the cells and release TF. The mixture was then dialyzed with stirring at 4 C for 48 hours with two changes of 50 ml sterile distilled water (final dialysate ratio of 100 to 1). The two 50 ml dialysates were pooled, lyophilized, resuspended in 5.0 ml saline, and injected intraperitoneally into normal Strain 13 recipients in a ratio of one donor to one recipient. Cells from recipient animals were cultured prior to receiving TF and one week after TF to determine changes in their capacity to respond to TSA by the lymphocyte transformation assay. In addition, recipient animals were skin tested one week after receiving TF (subsequent to bleeding for the lymphocyte culture assay) to assay delayed skin reactivity to TSA. Finally, all recipients were challenged with MCA-1 to determine their capacity to prevent tumor formation. Evidence of progressively growing tumors in recipients of immune or normal TF animals was recorded at 4-day intervals for a period of 6 weeks.

Analytical Disc Gel Electrophoresis: Polyacrylamide disc gel electrophoresis of serum was performed in 7% acrylamide gels at 1.5 mA/gel for 45 minutes according to the methods of Ornstein (111) and Davis (112) (Buchler Instruments, Inc.). In experiments where serum samples were sequentially collected from one animal, electrophoresis was performed at the same time (using approximately 0.8 mg protein/gel) to facilitate comparison. Significant variations were not observed when a single sample was electrophoresed on different days. The gels were stained for 5 minutes with 1% Amido-Schwartz in acetic acid (10% v/v), destained overnight in 10% acetic acid, and scanned with a densitometer (Densicord Model 542A, Photovolt Corp.). The percent of each globulin fraction compared to the total globulins (excluding albumin) was calculated by automatic integration of the gel tracing.

Preparative Disc Gel Electrophoresis: Preparative disc gel electrophoresis was performed in 10% acrylamide gels (5.0 mA) for 8-24 hours at 4 C (66) (Canalco, Inc.). Sample volumes containing 0.5-1.5 ml of either serum or KCl-extracted TSA were employed. Fractions were monitored by UV adsorbance (LKB Model 4700, LKB Instruments, Inc.) and collected in 10-20 ml fractions (Model 322, Instrument Specialities, Co.). Protein content was assayed by adsorbance at 280 mμ after concentration and dialysis under a vacuum (Prodicon, Bio-Molecular Dynamics).

Methods to Detect Antibody by Precipitation:

Gel diffusion - Ouchterlony two-directional gel diffusion in 1% Noble agar was performed on serum from tumor-bearing, immune and control

animals according to standard procedures (113). Three concentrations of TSA were used in this assay: stock TSA (20 mg/ml), 5-times concentrated TSA or a 1/5 dilution of TSA. Serum and TSA were added to the respective wells in sample volumes of 0.2 ml. All plates were incubated overnight, in a humid atmosphere, at 37 C.

Ring test - The ring or interfacial test was performed on serum from tumor-bearing, immune and control guinea pigs according to standard procedures (113). The same concentrations of TSA used above were tested in this system: 100 mg/ml, 20 mg/ml and 4 mg/ml. Serum samples (approximately 0.2-0.5 ml) were carefully overlaid with a similar amount of TSA. These volumes represented two to three mm in the glass tubing used (6 x 50 mm test tubes). The tubes were incubated at room temperature for 2 hours.

Passive Cutaneous Anaphylaxis (PCA):

Mice - Swiss-Webster mice were lightly anesthetized and injected intradermally with 0.025 ml of serum from tumor-bearing animals in various concentrations (undilute, or diluted 1 to 2 or 1 to 5). Control animals were injected with 0.025 ml saline. All experiments were performed in triplicate (3 mice per group). Animals from test and control groups were challenged intravenously in the tail vein 3 hours later with 0.2 ml of an equal mixture (v/v) of 1% Evans blue dye and TSA (100 mg/ml, 20 mg/ml or 4 mg/ml). PCA reactions were recorded 30 minutes later after sacrificing the animals and refecting their skin according to standard procedures (114,115). The antigen Ascaris suum (0.1 ml antigen + 0.1 ml Evans blue dye), and antiserum (0.025 ml)

directed against this antigen (prepared in mice) were used as a positive control (115).

Guinea pigs - Strain 13 guinea pigs were injected intradermally with 0.1 ml of serum from tumor-bearing animals in the same concentrations used above in the mouse system, or with serum from normal or MCA-1 immune animals. Three hours later, all animals were challenged intracardially with 0.5 ml of an equal mixture (v/v) of 1% Evans blue dye and TSA (100 mg/ml, 20 mg/ml or 4 mg/ml). After thirty minutes, the animals were examined for evidence of PCA (blue discoloring) at the serum injection sites. The animals were then sacrificed and the skin reflected at the site of the ID injection for closer observation. PCA reactions were then measured and recorded. The antigen Bovine Serum Albumin (BSA, 0.2 mg in 0.5 ml + 0.5 ml Evans blue dye) and rabbit anti-BSA antiserum (diluted (1/5, 0.1 ml) were used as a positive control system in the guinea pig.

Statistics:

Standard deviation:

The square root of the arithmetic average of the squares of the differences between observations and their mean.

$$S.D. = \sqrt{\frac{\sum(x-\bar{x}^{\,2})^2}{N-1}}$$

X = Sample observation
N = Number of observations

Standard error of the mean:

The standard deviation of the observations in the sample ÷ the square root of the number of observations.

$$S.E. = \frac{S.D.}{\sqrt{N}}$$

S.D. = Standard deviation
N = Number of observations

Correlation coefficient:

A measure of the degree of association found between two characteristics in a series of observations (on the assumption that the relationship between the two characteristics is adequately described by a straight line). Its value must lie between +1 and -1, either plus or minus 1 denoting complete dependence of one characteristic on the other, and 0 denoting no association between them. A plus sign shows that an upward movement of one characteristic is accompanied by an upward movement in the other; a negative sign that an upward movement of one is accompanied by a downward movement of the other.

$$r = \frac{\Sigma x_1 x_2}{\sqrt{\{(x_1^2)(x_2^2)\}}}$$

x_1, x_2 = variables, independent or dependent
r = correlation coefficient

Students t test:

A measure of deviation of the estimated mean ($\bar{x}$) from that of the population (y).

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\left(\frac{S_1^2 + S_2^2}{N}\right)}}$$

$\bar{x}_1$, $\bar{x}_2$ = sample mean of two populations

S_1, S_2 = standard deviation of two populalations

N = number of observations

RESULTS

The major goals of this research project are, first, to develop a tumor-model system in the guinea pig, and second, to investigate the influence of non-specific humoral factors on tumor growth. Initial experiments describe the development of the model and include a) the carcinogenic induction of tumors in the guinea pig and b) a description of the model in terms of growth rate, histology, malignancy, development of immunity and tumor specificity. In additional experiments, *in vivo* and *in vitro* assays for detecting host-protective tumor immunity will be evaluated. Following development of the tumor-model, experiments are described which were designed to determine the role of non-specific humoral factors on progressive tumor growth, i.e., factors which may aid in the escape of tumor cells from lymphocyte-mediated destruction. Included in this section will be experiments describing the detection, assay and specificity of these factors as well as their relationship to immunosuppression and tumor growth.

I. Development of the Guinea Pig Model:

Tumor induction, growth and passage - Three groups of Strain 13 guinea pigs, containing 12 animals per group, were injected subcutaneously in the abdominal region with one of three carcinogens, 3-methylcholanthrene (MCA), benzpyrene (BP), or dimethylbenzanthracene (DMBA). All animals were then palpated weekly for evidence of tumor growth. The first evidence of tumor growth appeared 3 months after carcinogen exposure in a BP-treated animal (Table 1). In this animal, a hard,

palpable, subcutaneous nodule in the region of carcinogen injection was observed and followed. The tumor grew progressively and the animal died 18 weeks later. This tumor was characterized histologically and passaged, as described later. The second and third tumors appeared 6 and 7 months after carcinogen exposure, in animals from the MCA- and DMBA-treated groups respectively. Six other animals from these three groups developed tumors during the 24-month observation period. All animals that developed tumors during this period were closely observed until death. A total frequence-of-induction rate of 25% was obtained for all treated animals in this experiment.

Tissues from the first tumor to arise in the three different carcinogen-treated groups were transplanted into 25 additional animals by trocar inoculation. Determination of tumor growth rates and evaluation of tumor passage by trocar inoculation were performed. Growth rates during the first in vivo passage were very similar for the DMBA- and BP-induced tumors (Fig. 2). Growth of these tumor-types was undetected for about 6-8 weeks, until they reached an average palpable size of approximately 1.0 cm. The average growth rate for both types was similar until the end of 14 weeks, when the average tumor size was 6-7 cm in diameter. During this time period, 20 of 25 and 21 of 25 animals from the BP- and DMBA-groups developed tumors. Variations in tumor size within these groups at any day of observation was less than 1 cm from the mean. Death occurred in all animals by the end of 15 weeks of tumor growth. Tumor regression was never observed.

Animals injected with MCA-induced tumor tissue (MCA-1) formed palpable tumors within 4 weeks of inoculation (Fig. 2). This latent

period was about half of that observed with the BP and DMBA tumors. The growth rate of the MCA-induced tumor was similar to that observed in DMBA- and BP-injected animals, after the tumors reached a 1.0 cm palpable size. By the end of the eighth week, the MCA-1 tumors reached an average size of 7 cm. By the end of the ninth week, 20 of 25 animals in which tumor growth was observed had died. As with the other two tumor types, the MCA-1 tumors never regressed after reaching a palpable size.

On the basis of the shorter latent period and subsequent earlier death of MCA-1 transplanted animals, the MCA-1 tumor line was chosen for further development for use in the guinea pig model. All experiments discussed henceforth, except those involving tumor specificity studies, employed the MCA-1 tumor line.

A consistent method for tumor passage was then sought which was less traumatic than trocar inoculation and which was more amenable to standardization in terms of numbers of viable cells injected. A single cell suspension (SCS) of MCA-1, prepared by differential centrifugation of a fine tumor mince, satisfied the above criteria. In all experiments, SCS was diluted with normal saline to contain 1×10^8 tumor cells/ml. Viability studies on selected preparations of SCS, using the method of trypan-blue dye exclusion, consistently demonstrated that 85-95% of the tumor cells were viable.

The effectiveness of SCS as a method for tumor passage was demonstrated in a preliminary experiment in which 11 animals were injected subcutaneously in each rear flank with SCS prepared from the eighth *in vivo* passage of MCA-1. Tumors were palpable in all animals within

11-14 days, reaching an average size of 5.5 cm within 4 weeks (Fig. 3, 4). All animals injected with SCS died within the following 4-7 days, with an average tumor size at the time of death of 5.8 cm. Comparing this with trocar inoculation from the same passage, SCS reduced the latent period nearly half and was 100% effective in passage success. In other experiments, a total of 147 normal Strain 13 guinea pigs, injected with a single cell suspension of MCA-1 prepared from different passages, had tumor growth rates similar to those mentioned in the above experiment. Furthermore, it should also be emphasized that whenever normal Strain 13 guinea pigs were injected with SCS, prepared from any passage of MCA-1, the success of tumor transfer was always 100%. The dose-response of SCS was determined from groups of animals receiving 10^4, 10^5, 10^6, 10^7 and 10^8 MCA-1 cells each. SCS with 10^6 cells or greater was uniformly lethal, while doses of 10^5 and 10^4 cells were not.

Histological characterization of MCA-1 - The gross and microscopic description of MCA-1 was the same on initial and on passage numbers 3, 8, 15 and 23. The tumor was light grey in color and appeared soft and spongy in texture and consistency. In tumor-bearing animals, MCA-1 was encapsulated and rounded in shape. Microscopically, the cells were fusiform to globular in shape and contained varying amounts of eosinophilic and slightly granular cytoplasm (Fig. 5,6,7). The nuclei, usually elongated or spindle-shaped, displayed occasional pleomorphic and hyperchromic variation. Mitotic figures were observed in all sections of tumor examined. MCA-1 was sarcomatous in appearance and classified as a moderately-differentiated malignant fibrosarcoma, expressing highly invasive characteristics.

Metastatic growth, although rare following SQ inoculation of MCA-1, was histologically similar. The tumor was classified as malignant and never regressed in normal Strain 13 guinea pigs after detection.

Stimulation of host-protective tumor immunity - The capacity of MCA-1 to stimulate host-protective, tumor immunity was evaluated by injecting tumor tissue into normal Strain 13 guinea pigs by various methods, including trocar inoculation, subcutaneous injection of tumor cell homogenates, and injections of fine tumor minces by subcutaneous, intraperitoneal and intradermal routes. The stimulation of immunity was then assayed by rechallenging surviving animals with an injection of 1.0 ml of SCS (1×10^8 cells/ml) in the rear flank region, a dose 100 times greater than that necessary to produce tumor formation in normal animals 100% of the time. Control animals were injected with 1.0 ml of SCS in the same way. All rechallenged animals were observed for 16 weeks.

Following an initial injection of MCA-1 by trocar inoculation, 16 of 22 animals survived and had no evidence of tumor formation after 16 weeks (Table 2). When these 16 remaining animals were rechallenged with SCS, however, only 6 of 16 (37.5%) were able to resist this tumor rechallenge. These 6 survivors were considered to be immune to MCA-1 tumor tissue. All normal control animals injected with SCS died within 5 weeks.

Six animals were inoculated with saline homogenates of MCA-1 (an equal amount of tissue used for trocar inoculation, 50% v/v, as described in Methods) by a subcutaneous route. None of the 6 animals showed evidence of tumor formation 16 weeks after injection (Table 2).

Upon rechallenge with SCS, however, only 1 of these 6 animals survived and was tumor-free 16 weeks after injection.

Experiments were also designed to stimulate host-protective tumor immunity by injecting finely-minced MCA-1 tumor tissue by different routes. The inoculum, consisting of 1.0 ml of minced tissue (step one of SCS preparation, as described in Methods) in saline (50% v/v), was injected by subcutaneous, intraperitoneal or intradermal routes. Of 44 animals injected by a SQ route with this inoculum, only 23 survived (Table 2). When these remaining 23 survivors were later rechallenged with SCS, all animals demonstrated tumor growth and died. Similarly, of 6 animals injected intraperitoneally with the finely minced inoculum, only one survived. This lone survivor was unable to resist a rechallenge with SCS. In contrast, however, of 14 animals injected by an intradermal routs, 13 survived and had no evidence of tumor growth. When these 13 survivors were rechallenged with SCS, 11 of 13 (85%) survived and were tumor-free 16 weeks later. These animals were rechallenged as many as 4 times with SCS and consistently resisted tumor formation. This experiment was repeated 5 times with identical results, using a total of 62 animals, and exhibited the same 85% immunization success rate. The intradermal procedure for immunization, with subsequent confirmation by SQ injection of SCS, was the method of immunization in all other experiments requiring immune animals.

After the intradermal method of immunization was proven effective, studies were conducted to determine specificity of the tumor immunity produced. To evaluate the degree of cross-reacting immunity produced by MCA-1 and tumors induced by other chemical carcinogens, 4 animals

each were immunized with MCA-1, DMBA or BP-induced tumor tissue by the intradermal method. Tumor growth was not observed, and 16 weeks later all animals were then rechallenged SQ with 1.0 ml of a single cell suspension (1×10^8 cells) of their respective tumor type to confirm immunity. Tumor growth was not observed in any of the animals for 16 weeks. Each animal from all three groups was then injected with 1.0 ml of SCS of each tumor type at different sites. In every animal, tumor growth was observed at the two sites injected with tumor tissue to which the animal was not initially immunized. Immunity produced by these different tumor types was not cross-reactive; the tumors did not regress and were lethal for their hosts within 5 weeks. No tumor growth was observed at the site of inoculation of SCS which was homologous to the immunizing inoculum.

Specificity studies were also designed to determine the degree of cross-reacting immunity produced by two, similarly-derived methylcholanthrene-induced tumor lines; MCA-1 and CMCA (derived by the same method by another investigator, as described in Methods). Three guinea pigs in each tumor-group were injected intradermally with finely-minced tumor (either MCA or CMCA) and rechallenged with the appropriate SCS as above, to produce and confirm the development of immunity to MCA-1 or CMCA tumor tissue. Animals from both groups were then challenged SQ with 1.0 ml of a SCS (1×10^8 cells) of MCA-1 tumor cells on the left side and CMCA tumor cells on the right side. Within 2 weeks, animals from both groups had palpable tumors on their right (CMCA) sides, but no evidence of growth on their left sides. Within 4 weeks, the CMCA tumors in the MCA-immune animals grew to a size of 1.5 cm, while the

CMCA tumors in the CMCA-immune animals grew to a size of 3.5 - 4.0 cm. At this time, there was still no evidence of tumor growth on the left (MCA-injected) side of any animal. Within an additional week, all tumors regressed and every animal appeared healthy. The animals did not form tumors on either side during the following 4 months and appeared to be in normal health. These experiments indicated that although immunity to the MCA-1, BP, and DMBA tumors was distinct, the MCA-1 and CMCA tumors showed a level of cross-reactivity.

Assays for tumor immunity - In order to detect tumor immunity without subjecting the host to tumor rechallenge with a single cell suspension of MCA-1 experiments attempting to extract MCA-1, tumor-specific antigens (TSA) were performed. Preliminary experiments, using saline extraction or homogenization methods, were unsatisfactory. The resulting preparation was unable to elicit delayed skin responses in known immune animals after intradermal injection of concentrated or diluted doses.

A hypertonic, salt-extraction procedure, initially designed to extract histocompatability antigens from human leukocytes, was then evaluated. This method, also modified to extract tumor antigens from guinea pig hepatomas, uses 3 molar potassium chloride (3M-KCl) dissolved in phosphate buffer pH 7.4. Preliminary experiments were designed to elicit delayed skin reactivity to MCA-1-extracted antigens, injected intradermally in several different concentrations. The results from these experiments demonstrated that TSA (20 mg protein/ml) elicited a mean ± S.D. delayed skin response of 7.7 ± 4.3 mm induration in 44

immune animals (Fig. 8) compared to a mean ± S.D. of 1.6 ± 2.5 mm induration in control animals ($P < .05$) (Table 3). Three tumor-bearing animals, also skin tested with the same antigen preparation, had a mean ± S.D. delayed skin test response of 3.7 ± 1.2 mm induration ($P < .2$). Higher concentrations produced nonspecific toxicity in control animals and lower concentrations were ineffective. In additional experiments, using other KCl-preparations of TSA, 12 other tumor-bearing animals had skin test reactions of the intermediate size (4.6 ± 1.4 mm induration, $P < 0.05$), when compared to controls.

Isolation of TSA was then attempted in order to obtain a preparation which would be less toxic to control animals and elicit larger or more intense skin test responses in immune animals. For this purpose, disc gel electrophoresis was employed using 12 λ (0.95 mg protein) TSA. In one preliminary experiment, saline eluates from 7 arbitrarily-chosen disc gel fractions were used to skin test 7 immune and 2 control animals (Table 4). A positive skin test (greater than 3 mm induration or erythema) was observed with the fourth fraction in all seven immune animals, but no response was observed with the fourth, or any other fraction, in control animals. In three immune animals, however, a positive, but less intense, response was noted in the sites injected with fractions 3 and 5. This experiment indicated that disc electrophoresis may be a useful procedure to isolate TSA which migrated in a region representing < 10% of the protein in the crude extract. The large amount of tumor tissue required to obtain a workable amount of material for evaluation, however, limited its usefulness. All subsequent experiments were performed with TSA isolated by only the 3M-KCl procedure.

Two in vitro procedures were then evaluated for their ability to detect cell-mediated immunity to MCA-1: macrophage migration inhibition (MMI) and lymphocyte transformation (LT). In preliminary MMI experiments, peritoneal exudative cells (PEC) from 15 of 17 immunized guinea pigs migrated only 20-40% as far as PEC's from normal animals when tumor antigen was present in the medium (TSA = 10% v/v, 2.0 mg protein/ml). Peritoneal exudative cells from immunized or normal control animals migrated with identical patterns when TSA was not added to the medium. A MMI response was considered positive if PEC's in the test system migrated less than 40% of that of control PEC's. Although this in vitro assay was capable of detecting immune reactivity of MCA-1 immunized animals to TSA, this procedure necessitated sacrificing the PEC donor animals. Consequently, the MMI assay was only used when animals were sacrificed during the course of an experiment.

The lymphocyte transformation assay was then employed to detect reactivity of peripheral blood lymphocytes from MCA-1 immune animals to TSA. A modified whole blood culture technique, originally reported in 1972 by Han and Pauly (109) was used. Preliminary experiments were designed to determine the level of lymphocyte transformation of guinea pig leukocytes stimulated with the mitogens phytohemagglutinin (PHA) or pokeweek mitogen (PWM). Dose-response experiments indicated that 5.0 μg of PHA-P and 1/200 dilution of stock PWM per tube were optimal for transformation, when 3.0 ml of a 1/30 dilution of guinea pig whole blood were used (Fig. 9). Each tube contained a mean ± S.D. of 19.7 ± 3.6 x 10^5 leukocytes diluted in medium RPMI-1640. The optimal culture period of this system was 6 days at 37 C (Fig. 10). All parameters were tested in triplicate.

More extensive studies were then performed to ascertain the average level of mitogen-induced stimulation in cultures from a large group of animals and to evaluate statistical variation within the system. In two separate experiments using 36 and 32 animals, lymphocyte responsiveness to PHA stimulation was a mean ± S.D. of 34,000 ± 4,000 and 52,000 ± 6,000 CPM respectively. Cultures from each animal were tested in triplicate. Similarly, the level of lymphocyte responsiveness to PWM was a mean ± S.D. of 46,000 ± 4,000 and 71,000 ± 5,000 CPM in the same respective animal groups.

The above results suggested that the whole-blood culture system could effectively measure lymphocyte transformation in the guinea pig. Attempts to apply this method to evaluate lymphocyte reactivity of MCA-1 immune animals to TSA, however, were not encouraging.

Initial experiments were performed in cultures from MCA-1 immune animals with various concentrations of TSA (0.002-200 mg protein per tube) in order to determine the optimal dose required in the culture system. The optimal response in these experiments at 20 mg TSA per tube was only 2-3 fold higher than the response in cultures without antigen. A larger series of experiments was conducted in which cultures from 26 MCA-1 immune animals were tested, as well as cultures from 4 tumor-bearing and 12 control animals, using 2.0 mg TSA/tube. One to four fold increases in incorporation of tritiated thymidine were again noted in 20 of 26 animals from the immune group (Table 5). However, similar increases were observed in cultures from both control (10 of 12) and tumor-bearing (4 of 14) animals. Control cultures, containing no TSA, had responses within a range of 400-1500 CPM.

Similar studies were repeated in 2 additional experiments, using other preparations of TSA with the same results. Lymphocyte transformation could not be used, therefore, as an *in vitro* assay to detect MCA-1 tumor immunity with TSA, because of the level of transformation in control cultures.

Collectively, the results evaluating *in vivo* and *in vitro* methods for detection of MCA-1 tumor immunity, demonstrated that delayed skin reactivity and inhibition of macrophage migration using TSA were the best indicators of immune reactivity to MCA-1.

Several assays using TSA were also employed to detect tumor-specific antibodies in immune or tumor-bearing animals. The precipitin (ring) test, Ouchterlony 2-directional gel diffusion, and passive cutaneous anaphylaxis (PCA) were carried out with several concentrations of TSA and serum from 3 immune or 3 tumor-bearing animals.

Precipitation in the ring test was not observed in any serum sample using stock TSA (20 mg/ml), 5-times concentrated TSA or a 1/5 dilution of TSA. Likewise, antibody against TSA could not be detected by the gel diffusion assay. PCA assays in either homologous (guinea pig) or heterologous (mice) hosts were unable to detect antibody reactivity to TSA. Again, immune or tumor-bearing animals were tested, using undiluted serum, and serum diluted 1 to 2 or 1 to 5 with saline, and the above concentrations of TSA. Following a 3 hour incubation period to allow skin-fixation of antibody, TSA was mixed with Evans blue dye (50% v/v) and injected intravenously. No reactions were observed with any serum and TSA combination. A control PCA assay in the guinea pig, using a BSA, anti-BSA antibody system, and in the mouse, using an

Ascaris suum, anti-Ascaris suum antibody system (as described in Methods) were positive. These results indicate that these methods could not detect anti-tumor antibody to TSA in MCA-1 immune or tumor-bearing animals.

In summary, a model system to study tumor immunity in the guinea pig has been developed and described. Experiments were designed to a) measure growth rates of chemically-induced tumors, b) determine the extent and characteristics of tumor malignancy, c) stimulate, detect and assay anti-tumor immunity, and d) determine specificity of anti-tumor immunity. The tumor line (MCA-1), chosen for development in this model, was induced by the chemical carcinogen, 3-methylcholanthrene (MCA), and grows to a lethal size in 4-5 weeks when normal animals are injected subcutaneously with 10^8 tumor cells. MCA-1 was classified as a malignant fibrosarcoma (moderately-differentiated), with highly invasive characteristics. The MCA-1 tumor line stimulates host-protective, tumor immunity to MCA-1 rechallenge by intradermal injection of viable tumor tissue. MCA-1 has distinct tumor specificity when compared to other chemically-induced tumor lines, but some degree of cross-reactivity with another chemically-induced tumor induced by the same carcinogen. Tumor-specific antibody was not detected in MCA-1 immune or tumor-bearing animals by the methods described. The guinea pig-tumor model, therefore, is a suitable model for the study of tumor immunity and for use in the second aim of this research project, i.e., investigation of the possible influence of non-specific humoral factors on tumor growth as a possible mechanism of escape of tumor cells from cell-mediated immune destruction.

II. Influence of Non-Specific Humoral Factors on Tumor Growth:

The second aim of this research project was to determine the role of non-specific humoral factors on progressive tumor growth, i.e., factors which may aid in escape of tumor cells from lymphocyte-mediated destruction. Included in this section are experiments describing the detection, assay and specificity of these factors, as well as their relationship to immunosuppression and tumor growth.

Detection of serum changes during tumor growth - For a number of years, investigators (100-102) have observed relative serum globulin changes in tumor-bearing animals of various species. These altered serum electrophoretic patterns were thought due to either an altered host response to the tumor or to tumor products. In either case, these experiments have indicated that serum globulin changes in tumor-bearing animals can be readily detected by analytical disc gel electrophoresis. Changes in serum globulins during tumor growth in the MCA-1 tumor-model were detected by comparing the relative serum globulin levels of tumor-bearing animals to the relative serum globulin levels of normal or tumor-immune animals. Relative serum globulin levels were determined by analyzing serum from these animals by analytical disc gel electrophoresis. Following electrophoresis of 8-12 lambda (approximately 0.7 mg protein) of serum, the gels were stained with amido-Schwartz and scanned by densitometry. The gel scans were then divided into ten distinguishable globulin fractions (excluding the albumin region) (Fig. 11), and the percentage of each region calculated by densitometric integration of the gel tracing. Control experiments demonstrated that relative serum globulin levels of ten control sera did not change when

electrophoresis was performed within a range of 2-20 lambda (0.14-1.4 mg protein).

In preliminary experiments, individual sera were analyzed from groups of 5 tumor-bearing, immune and control animals. The tumor-bearing animals had 4-5 cm bilateral tumors in both flanks. The immune group was immunized by an intradermal injection of MCA-1, and was resistant to second and third rechallenges of a single cell suspension of MCA-1 (1.0 ml, 1 x 10^8 cells). The fourth globulin region (F4) was elevated in animals bearing large, progressively growing tumors (Fig. 12). The mean globulin percent ± S.D. of this fraction was significantly increased from 14.4 ± 0.92 and 13.6 ± 1.3 in the control and immune groups to 18.9 ± 1.5 in the tumor-bearing group ($P < 0.01$) (Table 6). Following this observation, 28 frozen serum samples from tumor-bearing animals were analyzed retrospectively in the same manner. The same fourth globulin fraction was elevated in each of the 28 samples and the mean ± S.D. of this fraction was significantly increased (19.1 ± 2.2) when compared to both control or immune groups ($P < 0.01$) (Table 6). It was also observed that fractions 3 and 9 were significantly elevated and fraction 7 was significantly decreased in frozen serum samples. Changes in these fractions may reflect changes in lability of certain serum proteins during long (8-16 months), frozen storage or may be of significant interest.

Two separate experiments were then performed to determine which of the changes, F4 of both prospective and retrospective groupr or F3, 7 and 9 of the retrospective groups, were associated with tumor development and growth. In the first experiment, five animals were inoculated

subcutaneously with SCS (1×10^8 cells) and 3 animals served as controls. In the second experiment, 6 animals received MCA-1 tumor tissue in the form of SCS, and 2 animals served as controls. After collection of serum, samples (8-12 lambda, approximately 0.7 mg) were analyzed by disc gel electrophoresis prior to tumor challenge, and on days 4, 8, 12, 16, 24, 28 and 32 after inoculation. Although animals from both experiments expressed variations in levels of F4 on days 4 and 8, these variations were not statistically different from controls (Table 7, Fig. 13). On day 12, F4 levels significantly increased in 2 animals (Experiment I, animals 1 and 4). Animals in Experiment II did not demonstrate any statistical variation in F4 levels on day 12. On day 16, however, 4 of 5 animals in Experiment I had significantly increased F4 levels and animals in Experiment II were beginning to express similar fluctuations in levels of F4 noted above in Experiment I, on days 8 and 12. The mean ± S.D. F4 level of all tumor-challenged animals (18.5 ± 2.9), from both experiments, compared to control levels (15.7 ± 0.6) was significantly increased ($P < 0.05$) on day 16. On day 20, the F4 levels were still increased in the same 4 animals of Experiment I observed on day 16. In addition 4 of 6 animals of Experiment II had significantly increased F4 levels on day 16. The average F4 levels of all tumor-challenged animals (21.0 ± 4.2) compared to control animals (16.1 ± 1.5) were statistically elevated on day 20 ($P < 0.05$). Significant elevations of F4 levels were again observed in the serum of tumor-challenged animals on all subsequent test days, until the animals were sacrificed or died from the tumor.

At the same time serum samples were collected from these animals to determine levels of F4, the tumor size of each animal was measured to determine if increases in F4 and increases in tumor size could be correlated. Palpable tumors were observed in 1 of 11 animals on day 8, and 5 of 11 animals on day 12; however, only one of these animals (Exp. I, animal 1) had an elevated level of F4 on day 12 (Table 8). By day 16, however, 4 of 5 animals with elevated levels of F4 had palpable tumors. The remaining animal of Experiment I on days 16 or 20 without an increased F4, did not have evidence of tumor growth (animal 3). There was no relation between F4 levels and tumor size in animals of Experiment II on days 16 or 20. On the remaining days of the experiment (days 24, 28 and 32), however, large tumors and increased levels of F4 were observed in all animals until their death (Fig. 14). Linear correlation analysis was used to determine the statistical correlation between the concentration of F4 and tumor size in these experiments (Fig. 15). A correlation coefficient of + 0.9178 was calculated for all animals (including controls) in this experiment ($P < 0.001$). Disc gel fractions F3, 7 and 9 did not correlate with tumor size and showed fluctuations during tumor growth, but on any single day were not significantly different from controls. None of these fractions correlated with tumor size.

Detection of serum changes during sensitization to non-tumor antigens - The previous results demonstrated that F4 levels increased soon after exposure to MCA-1 tumor antigens and remained elevated during the period of rapid tumor growth. The results also indicated that the sustained increase might be due to continued exposure to replicating

tumor antigens. These hypotheses were then tested in non-replicating antigen systems in which antigens are subject to elimination by specific and non-specific mechanisms in the host. Experiments were designed to determine if similar serum globulin changes occurred in guinea pigs after exposure to three antigens known to stimulate cell-mediated immunity: dinitrofluorobenzene (DNFB), tuberculin (PPD) and allogeneic skin.

Serum samples were collected from Hartley guinea pigs prior to initial antigen exposure and on selected days during and after exposure. The serum samples (8-12 lambda; approximately 0.7 mg protein) were then analyzed by disc gel electrophoresis and the relative serum globulin levels evaluated by densitometry, as above. Nine distinguishable globulin regions were consistently observed in Hartley guinea pigs in these experiments, although more than one electrophoretic species was present in certain fractions (Fig. 16).

When animals were sensitized to DNFB, significant changes in the F4 level were noted on the second and third bleeding (days 3 and 6 during sensitization) (Table 9, Fig. 17). Fraction 4 increased from a relative mean ± S.D. percent of 10.2 ± 1.6 prior to treatment to 17.0 ± 3.4 and 17.1 ± 2.6 on days 3 and 6 during treatment, respectively. By day 17, this region had returned to normal levels (9.6 ± 1.0). These changes were significantly increased ($P < 0.01$), and easily observed in the gels prior to densitometry (Fig. 18). Significant changes were not observed in any of the globulin regions during the 3-week study period in control animals (Table 10), or in ten additional animals treated topically with the non-sensitizing irritants carbontetrachloride or

petroleum ether. No changes were observed in total globulin levels during treatments with DNFB or the non-sensitizing irritants.

Animals sensitized to Mycobacterium tuberculosis also showed a significant rise in F4. The increase, however, occurred at different times after immunization (Table 11, Fig. 17). The mean F4 level from any one bleeding after immunization did not significantly differ from controls or pre-immunization values. However, the mean ± S.D. of the highest values obtained after immunization (14.4 ± 2.7) was significantly different ($P < 0.01$) from controls (9.6 ± 1.0) and pre-immunization values (9.2 ± 1.7).

In allografted animals, a significant increase in F4 was observed when compared to autograft recipients. The change in F4 levels was restricted to 3 and 6 days after grafting and 4-6 days prior to rejection, as similarly noted during DNFB sensitization. The maximum increase (9.5 ± 1.6 to 15.3 ± 2.6, $P < 0.01$) occurred 6 days after allografting (Figs. 17,19).

From these experiments, it is apparent that changes in F4 are related to antigen exposure, whether the antigen is a contact sensitizer (DNFB), a bacterium (Mycobacterium tuberculosis), or a transplantation antigen (allogeneic skin). This provides evidence that the sustained increase in F4 during tumor growth may be a characteristic of replicating tumor antigens.

Lymphocyte suppression associated with tumor growth - Recent reports have demonstrated that non-specific humoral factors have immunosuppressive effects on both cell-mediated and humoral responses to a number of antigens. The possibility that these factors cause

suppression of cell-mediated tumor immunity, and thereby allow tumor cells to escape lymphocyte-mediated tumor destruction, was investigated in the MCA-1 guinea pig tumor model. Experiments were designed to determine if serum globulin increases, observed in tumor-bearing guinea pigs, were associated with lymphocyte suppression, measured by both *in vivo* and *in vitro* techniques.

Whole blood cultures were prepared from animals challenged with a single cell suspension of MCA-1 (1×10^8 cells) on days 0, 4, 8, 12, 16, 20, 24, 28 and 32 to determine *in vitro* lymphocyte responsiveness during tumor growth. The animals were the same as those used to determine F4 levels during tumor growth, in the experiments described above. Both PHA and PWM were used to assess the level of lymphocyte reactivity. Because considerable variation was observed between tumor-bearing animals when stimulated *in vitro* to PHA or PWM, lymphocyte suppression was evaluated by comparing CPM of tumor-bearing animals to both pre-inoculation and control values in Experiment I, and to control values alone in Experiment II. Pre-inoculation values in Experiment II were not recorded. In both experiments, lymphocyte responses of tumor-bearing animals were considered suppressed if their ability to respond was half of that of control animals. All control animals were age and sex matched. Linear correlation analysis was employed to evaluate the statistical relationship between lymphocyte responsiveness and levels of F4.

Marked suppression to PHA was noted sporadically as early as 4 days after tumor inoculation and became pronounced by 12 and 16 days (Fig. 20, Table 12). By day 24 all animals were suppressed and remained

so for the balance of the experiment. The correlation coefficient between PHA responsiveness and F4 levels was - 0.7917, $P < 0.001$. Similarly, suppression of PWM responses was observed as early as 4 days after inoculation and was somewhat sporadic until day 20 when most animals exhibited severe and permanent suppression (Table 13, Fig. 20). The correlation coefficient between PWM responsiveness and F4 levels was - 0.8291, $P < 0.001$.

The immunosuppressive effect of serum from tumor-bearing animals was then tested on two expressions of cell-mediated immunity in vivo: delayed skin reactivity and ability to reject tumor allografts. Initial experiments were designed to assess the effects of serum from MCA-1, tumor-bearing guinea pigs (Strain 13) on delayed skin reactivity of Hartley guinea pigs sensitive to both DNFB and PPD. Five animals sensitive to DNFB and PPD were injected intraperitoneally with 5.0 ml of pooled, tumor-bearing serum daily, for 3 days. Control animals, sensitive to these antigens, were injected with either saline (5 animals) or normal (pooled) Strain 13 serum (2 animals). On the last day of treatment, all animals were skin tested with DNFB on one side and PPD on the other side to determine their ability to express delayed skin reactivity. A positive DNFB skin-test response was recorded if the skin test site contained homogenous erythema. A positive PPD skin test was recorded if greater than an 18 mm area of induration was observed. Both criteria were the minimum responses observed in control or test animals prior to treatment. Only 1 of 5 animals treated with serum from tumor-bearing animals could express a positive skin test response to DNFB (Table 14). Similarly, only this same animal could respond

normally to PPD. The remaining 4 animals were unable to mount positive delayed skin responses to either of these antigens. These experiments strongly indicated that serum from tumor-bearing animals contained non-specific humoral factors capable of suppressing delayed skin responses to non-tumor antigens.

Additional experiments were designed to investigate the suppressive effects of serum from tumor-bearing animals on tumor allograft rejection. An MCA-1 (histoincompatible) tumor graft is routinely rejected in outbred Hartley guinea pigs. Acceptance or short term growth of MCA-1 tumor tissue in Hartley guinea pigs would, therefore, indicate suppression of cell-mediated allograft rejection. Two groups of 6 Hartley guinea pigs were injected, as in the previous experiment, with 5.0 ml/day of tumor-bearing serum or with normal Strain 13 serum for 3 days. On the first of three treatment days, both groups received SQ and IP injections of a SCS (1×10^8 cells) of MCA-1. Twenty normal Hartley and eight Strain 13 guinea pigs received injections of MCA-1 only, to serve as negative and positive controls, respectively, for tumor growth. All animals were palpated for evidence of tumor growth at 3-day intervals. Within 7 days after tumor challenge, 2 of 6 animals treated with normal serum and 3 of 6 animals treated with tumor-bearing serum had palpable tumors. All normal Strain 13 animals injected with only MCA-1 had palpable tumors (positive control). None of the 20 non-serum treated Hartley guinea pigs had tumors (negative control). By day 14, 5 of 6 animals treated with tumor-bearing serum had palpable tumors, while normal serum-treated and untreated Hartley groups had smaller numbers of animals with tumors (2/6 and 1/20

respectively) (Table 15). Within 21 days of tumor challenge, tumors in animals treated with either normal or tumor-bearing serum had almost completely regressed. On day 28, only 1 non-serum treated Hartley guinea pig had a palpable tumor, which eventually regressed. All Strain 13 guinea pigs which received only MCA-1 died by day 28. These experiments demonstrated that serum from tumor-bearing animals contained factors capable of suppressing or delaying cell-mediated rejection of tumor allografts, and that these factors may be present in normal serum, but in lower concentrations.

Lymphocyte suppression associated with stimulation to non-tumor antigens - As demonstrated previously, levels of F4 increased in guinea pigs during sensitization to 3 non-tumor antigens: DNFB, PPD and allogeneic skin. These F4 levels, however, subsequently decreased, concommitant with the development of immunity. A decrease in the same F4 fraction was not observed during growth of MCA-1 tumors in Strain 13 guinea pigs, a time in which lymphocyte responsiveness to PHA and PWM was severely compromised. It was of interest, therefore, to determine if lymphocyte responsiveness was compromised in Hartley guinea pigs during the temporary periods in which antigen-induced F4 levels were elevated. Initial experiments were performed in Hartley guinea pigs in which lymphocyte reactivity was assessed in vitro during sensitization to DNFB. Whole blood cultures were set up prior to DNFB sensitization, on the fifth day of treatment, and seven days after DNFB treatment (day 12). Test cultures were stimulated with the non-specific mitogens PHA, PWM, or Concanavalin A (Con A) or with antigen (purified protein derivative, PPD) and transformation recorded as counts per minutes of

^{3}H-thymidine incorporation. Control animals were untreated or treated with petroleum ether or turpentine to control for toxicity.

Lymphocyte responsiveness (mean ± S.D.) to PHA in animals prior to treatment was 34,000 ± 4,000 (36 animals) and 52,000 ± 6,000 (32 animals) in two separate experiments (Table 16, Fig. 21). Lymphocyte activity in unstimulated cultures was always less than 1,000 CPM. DNFB treatment (5 days) in half of the animals from each experiment (18 and 16 animals respectively) reduced the PHA response to 6,800 ± 1,800 (80% suppression) and 18,700 ± 5,600 (64% suppression) respectively ($P < 0.01$). Seven days after termination of DNFB treatment, marked recovery of PHA activity was noted (21,600 ± 4,000 and 42,300 ± 4,300 respectively). The second half of the animals from each experiment (18 and 16 animals respectively), treated with ethanol alone as a control, and animals treated with petroleum ether or turpentine, showed no decrease in PHA activity during the same period.

Lymphocyte responsiveness (mean ± S.D.) to PWM stimulation in the same experiments as above was 46,000 ± 4,000 and 71,000 ± 5,000 CPM prior to DNFB sensitization (Table 17, Fig. 22). After 5 days of DNFB treatment, suppression of 83% (7,800 ± 1,700 CPM) and 74% (18,500 ± 4,400 CPM) respectively was noted ($P < 0.01$). Seven days after termination of DNFB sensitization, PWM responses recovered to about 80% of initial values.

Lymphocyte stimulation to Con A during DNFB treatment was tested in a single experiment. The response (mean ± S.D.) of 32 animals prior to sensitization was 26,000 ± 3,000 CPM and was suppressed to 11,000 ± 2,400 CPM in 16 animals exposed to five days of DNFB treatment

(58% suppression, $P < 0.01$) (Table 18, Fig. 23). One week after termination of sensitization, the Con A response had recovered to 17,000 ± 2,000 CPM. Suppression of 16 control animals tested was not observed.

To evaluate the suppressive effects of DNFB sensitization on antigen induced lymphocyte transformation, 32 guinea pigs were immunized with Mycobacterium tuberculosis H37RA 45 days prior to DNFB sensitization. One half of the tuberculin sensitive animals were sensitized to DNFB and half served as controls. The lymphocyte response (mean ± S.D.) to PPD was 21,300 ± 3,800 CPM prior to DNFB sensitization in 32 animals (Table 19, Fig. 24). In 16 animals treated for 5 days with DNFB, the tuberculin response was suppressed to 4,800 ± 1,200 CPM (77% suppression, $P < 0.01$). One week after termination of DNFB sensitization, the PPD response recovered to 18,000 ± 3,000 CPM. Control animals did not vary in response during this period.

To assess the suppressive effects of DNFB sensitization on cell-mediated immunity in vivo, 15 PPD-sensitive guinea pigs from the previous experiments were skin tested with 5.0 µg PPD during DNFB sensitization. The skin test response on the fifth day of DNFB treatment was diminished when compared to responses prior to treatment and one week after treatment in both size (mm induration) and intensity (central necrosis) (Table 20). Collectively, these experiments indicate an association between F4 and suppression of cell-mediated immunity, assayed in vitro by lymphocyte transformation to mitogens and specific antigen. The suppression is non-specific and only apparent when F4 is elevated.

Preliminary attempts to prevent tumor growth with transfer factor - Stimulation of cell-mediated immunity with transfer factor (TF), a small, dialyzable molecule with a molecular weight of between 2,000-5,000, has previously been demonstrated in the guinea pig (108). Activity was assessed by both inhibition of macrophage migration and by delayed skin test reactivity. In addition, it was recently demonstrated that transfer factor also influences levels of F4 in guinea pigs (116). It was of interest, therefore, to determine if passive transfer of MCA-1 immunity with transfer factor results in host-protective tumor immunity or enhancement of tumor growth resulting from TF induced increases of F4. The effectiveness of transfer was evaluated by both in vitro and in vivo methods.

Transfer factor was prepared from lymphocytes from 14 MCA-1 immune guinea pigs and 4 normal Strain 13 guinea pigs. All immune animals were skin tested with TSA 3 weeks prior to isolation of TF to confirm immunity (Table 21). In addition, migration inhibition assays were performed on all immune and control donor animals on the day of transfer to test their immune reactivity to TSA in vitro. A 1 to 1 donor-to-recipient ratio was used in this initial experiment.

One week following intraperitoneal injection of TF, all recipients and control animals were skin tested with TSA. Delayed skin reactivity in untreated or normal and immune TF treated animals was variable and not able to distinguish recipients of immune TF (Table 22). Nine of 14 animals receiving immune TF responded equal to or less than animals from control groups. Only a slight increase of induration (2-3 mm) was

noted in 5 of 14 recipients of immune TF and was considered insignificant when compared to control reactions. After the skin tests were read, all recipients were challenged subcutaneously with a single cell suspension of MCA-1 (1×10^8 cells) in the rear flank. Within 10 days after tumor challenge, all control animals receiving normal TF developed palpable tumors at the injection site (Table 23). In addition, eight untreated control animals injected with MCA-1 also had tumors at the injection site. At this same time, however, 4 of 14 animals receiving immune TF did not have any evidence of tumor growth, and by day 15 after tumor challenge, one of these 4 recipients of immune TF still did not exhibit tumor formation. Furthermore, all animals in the control groups had tumors on day 15 which were greater than 3.0 cm in diameter, while only 9 of 14 animals receiving immune TF had tumors of this size. Interestingly, metastases were also observed on day 15 in 3 of the 14 recipients of immune TF. Metastases appeared in animals which exhibited delayed tumor onset and which had primary tumors less than 3.0 cm in size. Metastases were never observed in recipients of normal TF or in untreated control animals receiving only MCA-1. By day 30, all untreated control and both normal and immune TF treated animals had tumors. All animals died by day 34.

This preliminary experiment indicates that at donor to recipient ratios (1 to 1) lower than that required to consistently transfer cell-mediated immunity in the guinea pig (6 to 1), tumor immunity was not passively transferred (108). Three animals, however, displayed metastatic spread of MCA-1 tumor growth which is rarely observed (2 of 312) in untreated animals. This significant finding may be related to

stimulation of weak or short-lived cell-mediated immunity or TF-induced increases in F4, as previously suggested (116).

DISCUSSION

Within the last three decades, tumor immunology has developed into a distinct branch of immunology in quest of an understanding of malignancy. As a specialty, tumor immunology received major recognition in 1943, when Gross (1) reported that tumor tissue had the unique potential to stimulate tumor immunity in syngeneic hosts. Gross attributed the unique immunizing ability of tumor tissue to new antigens that developed during malignant transformation. Subsequent studies by Foley (4), in 1943, Prehn and Main (5), in 1957, and Klein et al (6), in 1960, supported Gross' observations and concluded that many types of tumors (both chemical- and viral-induced), possibly all types of tumors, developed new antigens different from normal host-tissue antigens. The immunity produced against these tumor antigens was capable of protecting the host from subsequent tumor challenge.

It was not clear, however, whether host-protection from the tumor was mediated by immune cells, humoral antibodies or both. Mitchison (22), in 1954, Klein and Sjögren (23), in 1960, and others (24,25) demonstrated that both cell-mediated and humoral immunity were produced in response to injections of viable tumor tissue, but that the cell-mediated component conferred host-protection. In addition, studies by the Hellströms and others (26,29,31), in the early 1960's, demonstrated that humoral antibodies were even capable of enhancing tumor growth, possibly by inhibition of cell-mediated destruction of tumor cells.

During this same period, other humoral factors, including tumor antigens and antigen-antibody complexes, were also implicated in

enhancement of tumor growth. Alexander and Currie (37), in 1973, postulated that all three types of specific enhancing factors (tumor antibody, tumor antigen or immune complexes) could play a role during progressive tumor development. They proposed that these factors could either inhibit the function of immune lymphocytes directly by combining with specific tumor antigen receptors (enhancing antibodies or antigen-antibody complexes), or indirectly by competing with tumor cells for specific receptors on immune lymphocytes (tumor antigens or antigen-antibody complexes).

Many investigators are currently evaluating the role of specific humoral factors as a possible explanation of how tumor cells escape cell-mediated immune destruction. This question is of major importance in tumor immunology and is currently being pursued in many laboratories worldwide (37,117). It has become apparent, however, that other factors, possibly non-specific in nature, may contribute to blocking cell-mediated tumor destruction. The involvement of non-specific suppressive factors in the escape of tumor cells from immune destruction has not been critically evaluated in the past because of the lack of suitable animal models. Although inbred strains of mice and rats are available, cell-mediated immunity is difficult to detect and quantitate in these species. In addition, spontaneous tumor development is common in certain strains of these species. Therefore, in order to investigate the influence of non-specific humoral factors on tumor immunity, a suitable animal-model system was needed. It was the aim of this project to (a) develop a suitable tumor model system in the

guinea pig to study tumor immunity, and (b) using the model, investigate the influence of non-specific humoral factors on tumor growth.

Experiments describing development of the guinea pig tumor model system will be discussed initially, followed by a discussion of experiments demonstrating that non-specific factors are associated with tumor growth and with suppression of cell-mediated immunity. The importance of these findings in tumor immunology will also be considered.

Development of the Guinea Pig-Tumor Model:

The Strain 13 inbred guinea pig was selected as the most suitable species for development of a tumor-model system. Although tumor-models are available in other species, the guinea pig is an animal in which cell-mediated immunity can be readily produced, detected and assayed. Furthermore, spontaneous tumor development has been observed in certain strains of mice, rats, chickens and other species currently available as tumor-model systems, but has not been observed in the guinea pig.

Two laboratories are presently engaged in studies involving inbred guinea pig-tumor systems (40,47). Unfortunately, the tumors used in these investigations are not consistently malignant. Additionally, the tumor-models are not available for study by other investigators. Consequently, the development of a guinea pig-tumor model, in which the tumor is uniformly malignant, received major emphasis in this project.

Three chemical carcinogens were chosen as likely candidates for inducement of malignant tumors: dimethylbenzanthracene (DMBA), benzpyrene (BP), and 3-methylcholanthrene (MCA). Within 3 months of injection, tumor development was observed in one animal injected with BP

(Table 1). After an additional latent period of 3-4 months, a tumor appeared in one animal from each of the DMBA- and MCA-treated groups. By the end of the experimental observation period of 24 months, 4 of 12 animals had a DMBA-induced tumor, 2 of 12 animals had a BP-induced tumor, and 3 of 12 animals had a MCA-induced tumor.

Low tumor-induction rates with these and other carcinogens (generally less than 50%) have also been observed in the guinea pig by other investigators (24,54). It was generally suspected by these workers that guinea pigs might have had natural cancer resistence or some type of natural anti-tumor factor in their serum (15,43,50), but studies to confirm these hypotheses were inconclusive (71). Since the chemical carcinogens employed in the present experiments were used in sub-toxic amounts for tumor induction (the guinea pig has a low toxicity threshold for many polycyclic aromatic hydrocarbons), low induction rates were probably related to the doses of carcinogen employed, rather to natural resistence in the guinea pig. A number of laboratories are currently evaluating the relationship between toxic and tumorigenic doses of chemical carcinogens (51).

The results from induction experiments using the carcinogens MCA, DMBA and BP, also point out the necessity of using a transplantable tumor system to study tumor immunity in the guinea pig, rather than a primary tumor system. Considerable variation occurred in both the tumor-induction period for the three tumor groups (Table 1), and in the initial tumor-growth rates (Fig. 2). With the first in vivo passage, the MCA-induced tumor line (MCA-1) displayed a latent period nearly half that of the BP or DMBA tumor types, and grew to a lethal size in

about half the time (Fig. 2). For these reasons, in particular because of the shorter tumor latent period, the first MCA-induced tumor to arise was used to develop the guinea pig-tumor model.

Histological characterization of the MCA-1 tumor line, both after the first passage, and during later in vivo passages, remained consistent. The tumor line was malignant, invasive, non-metastasizing, and classified as a moderately-differentiated fibrosarcoma. In addition, MCA-1 tumors never regressed once reaching a detectable size of 1.0 cm. Other tumors induced in the guinea pig with chemical carcinogens have been classified as liposarcomas (51,55), hepatomas (47), and adenocarcinomas (54), but have not been extensively characterized or developed. Furthermore, certain of these latter tumor-types vary considerably with regard to both malignancy and ability to metastasize following passage in vivo (47).

Experiments designed to standardize the tumor inoculum from passage to passage demonstrated that a single cell suspension (SCS) was most suitable. Dose-response studies using MCA-1 demonstrated that 1×10^6 tumor cells (85-95% viability) consistently produced tumors in all normal Strain 13 guinea pigs, when injected by a subcutaneous route. It was also noted that when animals were injected with SCS (1×10^8 cells), rather than by trocar inoculation, tumor growth rates were uniform and death resulted in all animals within a range of 4-5 weeks. This was an important consideration for maintaining consistency of experimental design. In addition, SCS was useful as a positive control for tumor production in experiments assaying tumor immunity.

The next step in development of the guinea pig model was to determine if the MCA-1 tumor line was immunogenic, i.e., if inoculation with tumor tissue resulted in the development of host-protective immunity to subsequent rechallenge with viable MCA-1. In a large series of experiments, Strain 13 guinea pigs were injected with various inocula of MCA-1 by different routes. After waiting for a period of 16 weeks, all animals that survived the initial injection of MCA-1 were rechallenged subcutaneously with SCS (1×10^8 cells) to determine if the initial injection stimulated immunity. Animals which did not exhibit tumor growth after rechallenge with SCS, a suspension which produced tumor formation in all normal guinea pigs, were considered to be immune to MCA-1.

In these experiments, subcutaneous inoculation of small pieces of tumor (1-2 mm) by trocar or injections of finely minced tumor tissue did not effectively stimulate tumor immunity (37.5% and 0% of the animals, respectively, survived rechallenge with SCS) (Table 2). Similarly, intraperitoneal or subcutaneous injections of fine tumor minces or tumor homogenates of MCA-1, respectively, did not stimulate host-protective tumor immunity (0% and 17.0% of the animals survived rechallenge with SCS). On the other hand, when animals were challenged intradermally with a fine mince of MCA-1, 11 of 13 (85%) survivors of the initial challenge rejected a SQ rechallenge with SCS. Additional rechallenges (as many as 4) with SCS did not result in tumor growth in these immune animals. The development of sustained immunity, not consistently observed in other guinea pig tumor systems, is an important

consideration when evaluating the effectiveness of immunization procedures, and the development of a tumor-model system.

These results demonstrated that intradermal injection of viable MCA-1 tumor tissue produced sustained, host-protective tumor immunity. In addition, the results also indicated that tumor inocula of tumor minces or homogenates were not effective in stimulating immunity by subcutaneous or intraperitoneal routes. Similar observations were reported by Gross (49), in 1943, when mice were injected by an intradermal route with chemically-induced tumors. In addition, he noted that normal mice injected with the same inoculum by intramuscular, intraperitoneal or subcutaneous routes demonstrated progressive tumor growth. Gross proposed that skin was a unique organ with a potential to limit tumor growth, possibly because of histiocytes with non-specific potential for destroying foreign cells. Alternatively, Rapp et al (47) proposed, in 1968, that intradermal implantation of tumor tissue in the guinea pig provided a unique environment necessary for stimulating immunity. It is possible that decreased potential for vascularization in the skin could account for the inability of tumor cells to escape cell-mediated immune destruction. It is still unknown, however, how or why intradermal injections of viable tumor tissue stimulate the development of immunity. Investigations in a number of laboratories are currently attempting to answer this question (51,52).

The demonstration of MCA-1 tumor immunity in the guinea pig then led to the question of whether the immunity produced was specific or cross-reactive. The degree of immune specificity was tested in guinea pigs immunized with MCA-1, DMBA, or BP-induced tumor tissue. Animals

immune to one type of tumor were challenged with a single cell suspension of all three tumor-types, at different injection sites. In every animal, tumor growth was observed only at the two sites injected with tumor tissue to which the animal was not immunized. The results from this experiment demonstrated that immunity produced to one type of chemically-induced tumor was indeed tumor specific.

In addition, specificity of tumor immunity was evaluated between two similarly-derived, methylcholanthrene-induced tumor lines, MCA-1 and CMCA. As above, animals immunized to one or the other tumor line were subsequently rechallenged with both types of tumor tissue. In this experiment, CMCA tumor tissue grew temporarily in both CMCA and MCA immunized animals, but then regressed. It should also be noted that the CMCA tumor is rejected in most normal animals following primary tumor challenge, possibly because of greater immunogenicity. MCA-induced tumor tissue did not grow in animals from either group. The results from this experiment indicated that there was a degree of cross-reactivity in the immunity produced by either of these tumor lines. In MCA immunized animals, the CMCA tumor may have been rejected because of both tumor and histocompatability antigen difference, i.e., the CMCA tumor line may have been derived from a substrain of Strain 13 guinea pigs which was histoincompatable with our strain of animals. In addition, because of different growth rates, the CMCA-induced tumor may have been a faster or more potent stimulator of tumor repressor substances resulting in inhibition of MCA-induced tumor growth. Evidence to support this possibility has not been reported. These results are in contrast to those reported by other investigators, in mice, rats and

guinea pigs, who demonstrated that immunity produced against tumors induced by the same carcinogen was non-cross-reactive (48,53,57,58). Zbar et al (56), however, recently reported the occurrence of antigenic shifts in diethylnitrosamine (DEN)-induced hepatomas in Strain 2 guinea pigs. In cross-challenge experiments similar to those described above, changes in antigenic specificity were observed in 4 of 6 DEN-induced hepatomas. A tumor line with distinct antigenic specificity gained the ability to cross-protect guinea pigs challenged with other DEN-induced tumor lines after continuous passage *in vivo*. Experiments are presently being conducted in the DEN-tumor system to determine the extent and stability of the observed antigenic drifts (56). In addition, more sensitive assays for detecting tumor antigens have recently demonstrated shared tumor antigenicity between other chemically-induced tumors (118).

Although the most common and direct method of assaying tumor immunity is by tumor challenge, two disadvantages have limited its value in the guinea pig. First, tumor challenge of suspected immune animals may result in tumor growth and thereby prevent further evaluation of the immune state. Second, immune animals challenged with tumors have to be observed over a long period of time to insure that tumor growth does not occur. Therefore, assays to detect MCA-1 tumor immunity *in vivo* and *in vitro*, without subjecting the host to tumor rechallenge, were evaluated. Tumor-specific antigens, extracted from viable tumor cells with 3 molar potassium chloride, were used in these assays, to circumvent the requirement for viable tumor-cell suspensions.

The soluble tumor-antigen preparations could be stored at 4 C for several months with no apparent change in activity. The tumor antigens could also be quantitated by estimation of protein concentration. Preliminary experiments attempted to distinguish MCA-1 immune animals from normal animals by delayed skin reactivity to the tumor antigens (TSA). Results from these experiments demonstrated that the TSA preparation could induce a delayed skin response (mean ± S.D.) of 7.7 ± 4.3 mm induration in 44 animals immune to MCA-1 tumor rechallenge, but only a delayed skin test response (mean ± S.D.) of 1.6 ± 2.5 mm induration in 8 normal control animals ($P < 0.01$) (Table 3). Positive reactions could be detected with as little as 2 mg of TSA injected intradermally. The large standard deviation in both groups represents the wide range of delayed skin reactions observed. Although some animals from the immune group had reactions no larger than control reactions, other animals from the immune group had reactions as large as 18 mm induration. It was also noted that the delayed skin test response of 3 tumor-bearing animals with the same TSA preparation was between the average responses observed in control and immune groups (mean ± S.D. = 3.7 ± 1.2 mm induration). The average skin test response of 12 additional tumor-bearing animals, using different KCl-preparations of TSA, was also between responses observed in control and immune groups (mean ± S.D. = 4.6 ± 1.4 mm induration, $P < 0.05$). This observation indicates that tumor-bearing animals are able to express some level of cell-mediated tumor immunity, even when bearing large tumors. The strength of the response, however, may represent the compromised ability of tumor-bearing animals to destroy progressively growing tumor cells. Similar

observations have been noted in many tumor systems in both animals and man (76,118). The mechanisms responsible for the suppressed response may involve specific or non-specific humoral factors recently implicated in enhancement of tumor growth (37,100,101,102).

Since delayed skin reactivity may be influenced by a variety of host factors, in vitro assays which would not be influenced by these factors were investigated. The migration inhibition test was evaluated as a method to detect immunity to MCA-1 in guinea pigs. Inhibition of migration of peritoneal exudative cells (PEC's) from immune animals was demonstrated when TSA was present in the medium, but no inhibition was observed when medium containing TSA was added to normal PEC's. Further, inhibition of migration of either immune or normal PEC's was not observed in the absence of TSA. Similar results have been observed in the DEN-guinea pig tumor system by Meltzer (67), and in the liposarcoma guinea pig tumor system by Suter (119), using tumor antigens isolated by salt extraction or sonication of tumor tissue.

There is one distinct disadvantage to using the migration inhibition assay, however, and that is the necessity of sacrificing the animals to obtain the PEC's. In future experiments, the indirect migration inhibition assay, not used in this study, may prove useful in bypassing the disadvantage of animal sacrifice. Because of this requirement, however, another in vitro assay of cellular immunity, lymphocyte transformation, was evaluated for ability to detect MCA-1 immune reactivity to TSA. A modified whole blood culture (WBC) technique, originally reported by Han and Pauly (109), was used in these experiments. The WBC-technique, using heparinized whole blood diluted

1 to 30 with medium RPMI-1640, was the only method available to detect guinea pig blood lymphocyte reactivity to mitogens or antigens. Although lymphocytes from spleen and lymph node tissue are capable of responding to mitogen or antigen stimulation, the disadvantage of sacrificing the donor animal to obtain these cells limits the usefulness of these cell sources and techniques. All cultures were set up in triplicate, using 4-5 x 10^5 lymphocytes per tube. TSA was used in a concentration range of 0.002 mg to 200 mg per tube, in initial dose-response experiments. A TSA concentration of 2.0 mg/tube was optimal. Initial experiments aimed at detecting immune reactivity of MCA-1 immunized guinea pigs with TSA (2.0 mg/tube) were disappointing. Incorporation of tritiated thymidine in cultures from immune animals ranged from background levels of 400-1500 CPM to 7000 CPM (4-5 times background) (Table 5). Cultures from control animals, however, also exhibited reactivity to TSA (range of tritiated thymidine incorporation was 1-4 times background). Further, WBC cultures from tumor-bearing animals also displayed reactivity to TSA, with a range of reactivity similar to the range observed in control cultures. It can be concluded from these results, therefore, that lymphocyte transformation, using TSA at an optimal concentration of 2.0 mg, cannot detect immune reactivity of MCA-1 immunized guinea pigs.

Several speculative explanations could account for the apparent low-level stimulation to TSA observed in cultures from immune, control and tumor-bearing animals. First, KCl-extracted preparations of TSA may have supplied nutrients leading to an increased level of metabolism by the cells during the 6-day (optimal) incubation period. Nutrients

might include non-tumor antigen protein, or denatured protein, or other substances in the extraction mixture. Second, TSA preparations could have been contaminated with non-specific stimulating compounds, such as endotoxin, which could increase the level of lymphocyte transformation in both test and control cultures. This alternative is unlikely, however, because TSA injected into guinea pigs by intradermal, subcutaneous, or intraperitoneal routes did not induce toxicity. In addition, similar antigen preparations from other tumors, from both animals (54, 55,59) and man (65), did not contain endotoxin activity. Third, a primary immune response may have occurred in the presence of TSA during the culture period. Although unlikely, newly exposed or activated antigenic determinants resulting from the extraction procedure could have led to the observed increase in tritiated thymidine incorporation of control cultures, as well as cultures from MCA-1 immune or tumor-bearing animals. It is not known if any, or all, of these explanations account for the observed increases in control cultures.

This assay of immune reactivity, therefore, does not correlate with other assays used to detect immunity to MCA-1 in our hands. Similar conclusions have been reported by other investigators (120,121), i.e., lymphocyte transformation does not correlate with other assays of cell-mediated immunity. However, Meltzer (67), in the DEN-tumor system in guinea pigs, also observed 2-3 fold increases in lymphocyte transformation, using lymphocytes isolated from a preparation of peritoneal exudative cells and KCl-extracted tumor antigens. Control cultures in this system, however, did not respond to the antigen preparations using a wide dose range. This difference in lymphocyte transformation to

tumor antigens may reflect differences in the source of lymphocytes, since others have found that only PEC's can respond in this assay to tumor antigens (118).

To conclude, in the MCA-1 tumor system, soluble tumor-specific antigens were tested in 3 bioassays of cellular immunity, delayed skin reactivity, macrophage migration inhibition and lymphocyte transformation. The antigens were effective and specific in eliciting delayed skin test reactivity *in vivo*, and were capable of inducing macrophage migration inhibition *in vitro*. Lymphocyte transformation, another *in vitro* assay of cellular immunity, did not correlate with skin test reactivity or migration inhibition. The antigens were tumor specific and elicited reactivity at comparable antigen concentrations (approximately 2.0 mg protein). All MCA-1 immune animals were immunized intradermally with viable MCA-1, prepared as a single cell suspension, and were not previously exposed to the soluble tumor antigen extract. TSA was thus able to detect immunity stimulated by whole tumor cells, confirming its specific biological activity. TSA, therefore, provides a convenient means to evaluate the development of MCA-1, tumor-specific immunity, and effectiveness of various immunization procedures.

Humoral immunity was also assayed in MCA-1 immune animals using TSA. Attempts to detect humoral antibody using antibody precipitation procedures and the passive cutaneous anaphylaxis assay, however, were negative. This may have been due to the inability of the tests used to detect low levels of antibody or to the presence of antigen-antibody complexes. The inability to detect antibody in tumor-bearing or immune guinea pigs does agree, however, with results from other laboratories (122).

In summary, the MCA-1 tumor model in Strain 13 guinea pigs is a useful system to study tumor immunity. A number of characteristics about the model reflect advantages when compared with other existing tumor systems. First, the guinea pig itself is a convenient animal to use in the laboratory and does not exhibit spontaneous tumor development. In addition, passive transfer techniques for detecting or transferring immunity with transfer factor have been adequately described in the guinea pig. Second, the tumor used in this model, a 3-methylcholanthrene-induced fibrosarcoma (MCA-1), is highly invasive, never regresses after reaching a size of 1.0 cm and is uniformly malignant. Other guinea pig tumor models available vary in malignancy from passage to passage. Third, antigenic drifts noted in other guinea pig tumor systems have not been observed in the MCA-1 tumor model. Fourth, the tumor is 100% lethal when injected subcutaneously as a single cell suspension (1×10^8 cells) in all normal animals. This is a necessary positive control when attempting to detect or confirm immunity by direct tumor challenge. Fifth, immunity produced to MCA-1 is tumor-specific, protective, and does not diminish with time. Sixth, MCA-1 immunity can be assayed *in vivo* by delayed skin reactivity and *in vitro* by migration inhibition, using KCl-extracted tumor antigens. Collectively, the above characteristics of the MCA-1 tumor model are essential when evaluating various procedures to stimulate, detect and measure tumor immunity. Since some, or all, of these characteristics are lacking in other guinea pig models, this MCA-1 tumor system will hopefully allow further investigation of methods to stimulate and assay immunity. Further, since MCA-1 induced immunity and tumor growth have now been

evaluated, an investigation can be made of the mechanisms by which tumor cells escape from cell-mediated immune destruction.

Influence of Non-Specific Factors on Tumor Growth:

Recently, non-specific humoral factors have been implicated in suppression of cell-mediated and humoral immunity to a number of antigens. Cell-mediated tumor immunity may also be influenced by these factors, resulting in the escape of tumor cells from lymphocyte-mediated tumor destruction. With the use of the MCA-1 guinea pig-tumor model, the objective of the second phase of this research project was to determine if non-specific suppressive factors could be detected in tumor-bearing hosts, if they were related to tumor growth, and if they had the potential to suppress cell-mediated tumor immunity and lead to the escape of tumor cells from immune destruction.

Preliminary experiments in this project were based on previous studies in which "alpha globulin" changes were observed in serum from tumor-bearing animals (101,102). Our experiments in the MCA-1 system attempted to determine if similar changes could be detected in tumor-bearing guinea pigs, and if those changes were associated with tumor growth. The results from these experiments demonstrated that serum globulin changes could be detected in animals bearing large, MCA-1 induced tumors (Table 6). Significant elevations in one of ten electrophoretically-distinguishable globulin fractions (fraction 4, F4) were detected in serum from tumor-bearing animals, but not in serum from normal or tumor-immunized animals.

Experiments were designed to correlate the increases in F4 with MCA-1 tumor size, and determine if the increases were related to progressive tumor growth. Results from these experiments demonstrated that F4 increased to levels significantly different from control F4 levels within 12-16 days of tumor challenge in most animals (Table 7). Furthermore, it was observed that the increase in F4 was directly correlated with the progressive increase in tumor size (Table 8). In addition, F4 levels in all tumor-bearing animals remained at a significantly increased level during the last 2 weeks of life (Table 8, Fig. 14). Significant changes in F3, 7 and 9-associated tumor growth were not observed. These results strongly indicated that challenge with the MCA-1 tumor (tumor-specific antigens?) was responsible for the increase in F4.

These results also indicated that the MCA-1 tumor tissue (tumor-specific antigens?) was responsible for the increase in F4 in these animals, and that the sustained increase in this globulin fraction may have been related to continued (tumor) antigen exposure. This suggested that non-tumor antigens might also stimulate an increase in F4. In addition, since most non-replicating antigens have a relatively short half-life due to immune and non-immune elimination mechanisms, one might then expect F4 to return to normal levels when the antigen is no longer available. These hypotheses were tested in non-replicating antigen systems using dinitrofluorobenzene (DNFB), tuberculin (PPD), or allogeneic skin as the antigen source. In the Hartley strain of guinea pigs, exposure to any one of these antigens induced an increase in the same globulin fraction (F4) that was observed in the tumor

antigen system. Further, F4 then decreased to pre-exposure levels, concommitant with the development of immunity. In the DNFB and allograft sensitized animals, F4 increased 3-6 days after initial antigen exposure, but subsequently returned to presensitization or control levels by day 10 (Fig. 17). An increase in F4 was also observed during sensitization to tuberculin, but the increase developed over a broader time period when each animal was considered individually (Fig. 17). This may reflect differences in the antigen, variation in the sensitization process itself, or the use of Freunds' adjuvant.

The combined results from the above experiments demonstrated that F4 levels increased soon after antigen exposure, regardless of whether the antigen was capable of self-replication (tumor systems) or not capable of self-replication (DNFB, tuberculin, allogeneic skin). It appeared, therefore, that increases in F4 were related to recognition of a foreign molecule (antigen) and were common to all antigens tested. The important consideration, however, seemed to be the efficacy of the antigen, i.e., whether or not it was capable of self-replication. With continued antigen exposure, most probably due to the expression of tumor-specific antigens (TSA) in the above system, F4 levels were maintained at an elevated level. On the other hand, once non-replicating antigens (DNFB, PPD, or allogeneic skin) were removed from the host or degraded, F4 returned to normal levels. It is not known at this point if antigen induced increases in F4 represented activation of a humoral component normally in an inactive form, if a normal host constituent was produced in increased amounts during antigen exposure, or if an entirely new product was synthesized during exposure to antigen.

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An important point from the above results is that increased levels of F4 were observed in a malignant tumor system. If F4 represents the same immunosuppressive "alpha globulin" fraction that Mannick et al (82), Cooperband et al (88) and Riggio et al (89,90) isolated from the serum of normal animals and man, then F4 also may suppress cell-mediated tumor immunity. This hypothesis might then explain the apparent ability of MCA-1 tumor cells to escape lymphocyte-mediated tumor destruction. In short, immunosuppressive F4 levels would be increased in tumor-challenged guinea pigs during early exposure to tumor antigens. The immunosuppressive nature of F4 might then compromise cell-mediated tumor immunity and allow survival and growth of MCA-1 tumor cells. During growth of MCA-1, then, continued antigen exposure from increasing numbers of tumor cells could maintain immunosuppressive F4 at an elevated level, allowing progressive tumor growth.

Experiments were designed to test this hypothesis, i.e., to determine if MCA-1 induced increases in F4 were associated with suppression of lymphocyte responsiveness. Periodic evaluations of lymphocyte responsiveness by non-specific mitogen stimulation in vitro were then made during tumor growth. Levels of F4 were also monitored during the same periods. Results from these studies demonstrated severe and permanent suppression of both PHA and PWM-induced lymphocyte responses during the last 2 weeks of life in these animals (Table 12,13; Fig. 20). F4 levels during this period increased dramatically in all animals. Correlative analysis established that during progressive tumor growth, there was an inverse relationship between lymphocyte function and levels of F4 (PHA system: $r = -0.7917$, $P < .001$; PWM system: $r = -0.8291$,

$P < 0.001$).

These studies offered convincing support to the hypothesis that tumor-antigen induced elevations of F4 during tumor growth suppress cell-mediated tumor immunity, thereby allowing further development of the tumor. Other experiments also lend support to the immunosuppressive potential of serum from tumor-bearing animals. In these studies, the effect of MCA-1 tumor-bearing serum on delayed skin test reactivity was determined in animals previously immunized to both dinitrofluorobenzene (DNFB) and tuberculin (PPD). Normal or tumor-bearing serum was injected intraperitoneally into Hartley guinea pigs for 3 consecutive days. Following the last injection, animals from both treatment groups were skin-tested with DNFB or PPD. Results from these studies demonstrated that animals treated with tumor-bearing serum could not mount normal delayed skin responses to an intradermal injection of PPD or topical applications of DNFB (Table 14). Conversely, suppression was not observed in animals treated with either normal Strain 13 guinea pig serum or saline. The results indicated that serum from tumor-bearing animals contained non-specific factors capable of suppressing delayed skin test responses of animals sensitive to other (non-tumor) antigens. These factors were expressed in vivo following challenge with a variety of non-tumor antigens (DNFB, PPD, allogeneic skin) and tumor-specific antigens. They seemed to function non-specifically since they suppressed the expression of cell-mediated immunity to antigens which induced their appearance and to unrelated antigens.

A further evaluation of the non-specific suppressive nature of tumor-bearing serum was tested by attempting to inhibit the trans-

plantation response of Hartley animals challenged with MCA-1 tumor tissue. Results from this experiment indicated that serum from MCA-1 tumor-bearing animals was able to temporarily suppress the transplantation (histoincompatability) response of Hartley guinea pigs injected with allogeneic tumor tissue (MCA-1) (Table 15). All animals, however, were eventually able to overcome the effects of early serum treatment and reject the transplants. The largest number of animals with demonstrable tumor formation were observed in the group treated with serum from tumor-bearing animals. Some tumor formation, however, was noted in the normal serum-treated group, suggesting that normal serum might also contain certain levels of suppressive materials which only become increased during antigen exposure.

The results above confirmed that non-specific factors appear in increased levels during early antigen exposure and that, depending on the persistence of the antigen, either remain at elevated levels or subside. In addition, F4 levels were directly correlated with both increase in tumor growth and decrease in lymphocyte responsiveness to antigens which induced the increase in F4, or to other antigens. Experiments were then designed to determine if the transient F4 increases observed in animals during sensitization to a non-replicating antigen (DNFB) were also associated with immunosuppression. During sensitization to DNFB, lymphocyte reactivity to mitogen or specific antigen induced stimulation was suppressed at the time F4 levels were increased (Tables 16-18, Figs. 21-23). Responses were measured <u>in vitro</u> by lymphocyte transformation induced by the mitogens PHA, PWM and Con A.

Results from these experiments demonstrate that during DNFB sensitization, lymphocyte function was suppressed, but recovery occurred soon after the 6-day sensitization period was completed. During the period of lymphocyte suppression, F4 levels were increased, but then decreased to normal levels when immunity to DNFB developed. Correlative evaluation of the data confirmed the inverse relationship between mitogen-induced lymphocyte transformation in vitro and F4 levels in vivo.

Specificity studies were performed by examining lymphocyte function in tuberculin-immunized animals during sensitization to DNFB. The results from these studies demonstrated that during DNFB sensitization in animals preimmunized with Mycobacterium tuberculosis, lymphocyte responsiveness to tuberculin (PPD) was suppressed (Table 19). Furthermore, this same effect was observed in vivo, during DNFB sensitization, delayed skin reactivity to PPD was significantly reduced in both size and intensity (Table 20).

To summarize, the above experiments have demonstrated that exposure to both replicating and non-replicating antigens caused an increase in one serum globulin fraction (F4) in two strains of guinea pigs. F4 was directly correlated with increases in MCA-1 tumor size in Strain 13 animals, and with sensitization to non-tumor antigens in Hartley animals. The relationship between antigen exposure and immunosuppression was also confirmed in this project. Antigen-induced elevations in F4 were inversely related to the ability of guinea pigs to respond immunologically to non-specific mitogens in vitro and specific antigens in vivo. The mechanism of suppression, although still unknown might occur

at the level of antigen processing (macrophage level?) or at the level of lymphocyte activation (B or T lymphocyte level). In the DEN-guinea pig tumor system, Bernstein et al (123) proposed that impaired cellular immunity associated with progressively growing hepatomas might be due to macrophage dysfunction. Since macrophages were present in our in vitro assays (macrophage migration inhibition and lymphocyte transformation) of cell-mediated tumor immunity, it is possible that F4 may act at the macrophage level of developing immunity. On the other hand, suppression of PHA and PWM-induced lymphocyte responses may occur at the lymphocyte level. Both T (thymus-derived) and B (bursal-derived) lymphocytes may have been affected in these experiments since PHA and PWM have been reported to stimulate these two cell types, respectively. Although recent investigations have indicated that PWM may also stimulate T cells, additional experiments using other T or B cell mitogens, and other methods to distinguish between these two cell-types, are required to establish which cell type or types are suppressed in the MCA-1 tumor system.

Recent experiments have indicated that immunity to non-tumor antigens can be effectively transferred to normal guinea pigs with transfer factor (108,124). Since levels of F4 might also be influenced by transfer factor (116), the transfer of "tumor immunity" with transfer factor might result in either stimulation of cell-mediated immunity and host protection from tumor challenges or an increase in suppressive levels of F4, leading to enhanced tumor growth. To investigate these possibilities, preliminary experiments to passively transfer tumor immunity from MCA-1 immune donors to normal recipients were attempted.

Recipients received transfer factor in a 1 to 1 ratio from MCA-1 immune animals, and were than assayed for transfer of immune reactivity by delayed skin test reactivity to tumor-specific antigens (TSA), and resistence to a challenge of viable MCA-1 tumor tissue. The results from these preliminary experiments were inconclusive, but indicated possible directions for future evaluation of cancer immunotherapy with transfer factor. In general, transfer of delayed skin test reactivity in most recipients was negligible (Table 22). There was no apparent correlation between degree of skin reactivity of donor animals and skin reactivity of their respective recipients, tested one week after transfer. In addition, the degree of tumor-challenge resistance, in recipient animals was also difficult to evaluate, but nevertheless provided information subject to interpretation. Within 10 days after tumor challenge, 4 of 14 animals receiving immune TF did not exhibit tumor growth, while all animals receiving normal TF and all untreated control animals had demonstrable tumor growth (Table 23). Additionally, 5 of 14 recipients of immune TF exhibited retarded tumor growth (< 3.0 cm in size) compared to control animals or animals receiving normal TF (> 3.0 cm in size) when measured 15 days after tumor challenge. Finally, an unexpected occurrence was observed in 3 of the 5 animals mentioned above with retarded tumor growth at the injection site. In these animals, metastatic tumor growth at sites far removed from the primary site was observed on the 15th day after tumor challenge (Table 23). Metastases were never observed in recipients of normal TF or in untreated controls receiving only MCA-1.

These results, although not conclusive or subject to statistical evaluation, require comment. Since it appears that with a low (1-1) donor-to-recipient transfer ratio tumor growth is only retarded in a small percentage of animals at best, higher transfer ratios (6 to 1) may delay growth longer or even prevent growth. Although higher transfer ratios are required for transfer of chemical sensitivities in guinea pigs (108), these preliminary experiments helped to establish baseline dose-response relationships in the MCA-1 tumor system. Further, the low donor-to-recipient transfer ratios allowed possible expression of TF-induced influences of F4 in recipient animals, since TF was probably not used in optimal amounts to transfer immunity. The metastatic tumor growth observed with these low transfer ratios may be of possible clinical significance. Although these findings have not been reported in other tumor systems, several explanations can be offered to account for TF-associated metastatic growth. First, transfer factor could contribute to metastatic spread by inducing an immune response at the primary injection site. During immune destruction of tumor cells at this site, an occasional tumor cell may escape by a vascular route, leading to metastatic growth at sites far removed from the primary tumor site. Second, TF preparations may contain other dialyzable factors of low molecular weight which can suppress (F4?) or compete (metastatic promoting factor?) with its ability to confer immunity (116). Third, transfer factor itself may stimulate increases in F4 and thereby directly compete with its ability to confer immunity. It is not known if any or all of these explanations could account for the metastatic growth observed, but future experiments measuring levels

of F4 after transfer may help clarify this observation.

In conclusion, experiments with the MCA-1 tumor model point to the possible involvement of a non-specific suppressive mechanism in the escape of tumor cells from cell-mediated immune destruction. Although other mechanisms may also account for enhancement of tumor growth, non-specific factors in the serum of tumor-bearing animals should now be seriously considered as a possible explanation.

Implications, Importance and Future Directions of this Research:

Of particular importance to tumor immunology are the observations demonstrating a direct correlation between tumor size and levels of disc gel fraction 4 (F4) in the serum of tumor-bearing guinea pigs, and an inverse correlation between F4 levels and lymphocyte responsiveness measured both *in vivo* and *in vitro*. These observations imply that the immunosuppressive factor(s) present in F4 has the potential to compromise cell-mediated tumor immunity and thereby allow tumor cells to escape destruction by the immune system. Results from this research project demonstrate that the immunosuppressive moiety in F4 is expressed after exposure to several types of antigens: those capable of self-replication (tumor antigen) and those incapable of self-replication (DNFB, PPD, allogeneic skin). An important difference in the suppression caused by these two general types of antigens, however, is that non-replicating antigens cause transient effects while replicating antigens cause continued immunosuppression (sustained elevations in F4 levels). This characteristic of tumor antigens may partially explain

why tumor cells continue to replicate in the face of demonstrable immunity and eventually become lethal.

These observations are of particular interest because of their diagnostic potential. Future studies should be expanded in the guinea pig using MCA-1 and other tumor lines, and in other animal tumor systems (both primary and transplantable) to evaluate the feasability of detecting tumor development by monitoring levels of F4. Investigations should be designed to measure F4 levels at more frequent intervals and at earlier times during tumor development than in the studies reported in this project. Assay systems with greater sensitivity than analytical disc gel electrophoresis, however, may be needed to detect and measure early F4 changes. A radioimmunoassay system, for example, may be useful in this respect, although it should be noted that this assay requires isolation of the active moiety in F4 for use in preparing reagents.

In addition, future studies should be designed to determine the site and mechanism by which the active moiety of F4 expresses its immunosuppressive effect. Results from this project indicate that F4 acts on the cell-mediated arm of the immune response (T cell level?) since both PHA and PWM responses were suppressed (both lectins stimulate T cell activity). Major emphasis should be placed on defining which component of the cell-mediated immune process (macrophage, T lymphocyte, others?) is compromised by F4. This could be done by studying each cell type separately in vitro. For example, kinetic studies with these cell types in the presence of F4 or its active

moiety (added prior to, concurrent with or following antigen or mitogen challenge) may indicate whether the activation phase or the effector phase of the immune response is involved. Similarly, isotype studies with F4 using immuno-labeling techniques may indicate where the factor binds to the attacking (or target?) cell. Studies may also be designed to investigate whether F4 is specific for lymphoid cells or whether it is also able to modify the metabolism of other cells. Measurement of nucleic acid or protein synthesis of normal or tumor-derived tissue culture cells may be beneficial in this respect.

Future studies may also be designed to further explore the relationship between F4 and transfer factor. Because both these factors appear to be small, dialyzable molecules of considerable clinical importance, investigations should determine if the presence of both factors in a single preparation negates the effects of the other (immunopotentiator capability of transfer factor or the immunosuppressive potential of F4).

It is apparent that many avenues are open to further examine the non-specific immunosuppressive nature of F4. It is hoped that this research will stimulate further interest and lead to greater insight into the mechanism by which tumor cells avoid destruction by the immune system.

SUMMARY AND CONCLUSIONS

In summary, this research project has described experiments designed to a) develop a suitable tumor-model system in the guinea pig to study tumor immunity and b) investigate the influence of non-specific humoral factors on tumor growth. The tumor line MCA-1, induced by 3-methylcholanthrene (MCA), was used in developing the tumor model. MCA-1 grows to a lethal size in 4-5 weeks in normal animals, following subcutaneous injection of 10^8 tumor cells. This tumor was classified as a malignant, invasive, non-regressing fibrosarcoma, which stimulates host-protective, tumor immunity following intradermal injection of viable tumor tissue. The immunity produced against MCA-1 was tumor specific when compared to other chemically-induced tumor lines, but somewhat cross-reactive to tumors induced by the same carcinogen. Tumor-specific antibody was not detected in MCA-1 immune or tumor-bearing animals by the methods described.

This model system was used to investigate the role of non-specific humoral factors which might function in the escape of tumor cells from immune destruction. Serum globulin elevations (Fraction 4, F4) were detected by polyacrylamide disc gel electrophoresis in guinea pigs, following exposure to both replicating (MCA-1) and non-replicating (dinitrofluorobenzene, tuberculin, allogeneic skin) antigens. Fraction 4 increases were found to be directly correlated with progressive tumor growth (MCA-1) and lymphocyte responsiveness in Strain 13 guinea pigs was found to be negatively correlated with concentration of F4 in the serum. In addition, globulin fraction 4 became increased in

Hartley guinea pigs during sensitization to dinitrofluorobenzene, tuberculin and allogeneic skin. The increase in F4, induced by both replicating and non-replicating antigens, was associated with suppression of lymphocyte responsiveness both *in vivo* and *in vitro*. Collectively, these experiments demonstrate the possible involvement of a non-specific suppressor mechanism in the escape of tumor cells from lymphocyte-mediated immune destruction.

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Table 1

Tumor Development in Inbred Guinea Pigs

Carcinogen[a]	Number of Animals	Animals with Tumors[b]	Induction Period (months)[c]
DMBA	12	4	7, 9, 15, 24
BP	12	2	3, 8
MCA	12	3	6, 13, 17

[a] Animals were injected subcutaneously in the abdominal region with 20 mg of dimethylbenz[a]anthracene (DMBA), 3,4-benzpyrene (BP) or 3-methylcholanthrene (MCA) dissolved in benzene.

[b] Animals in all three groups were examined weekly for evidence of tumor growth.

[c] The induction period of each tumor which was recorded during the 24 month observation period.

Production of Tumor Immunity in Strain 13 Guinea Pigs

Initial Tumor Challenge[a]			Subcutaneous Rechallenge with SCS[f]	
Method	No. Animals	No. Survivors	No. Survivors	% Survivors
SQ Trocar[b]	22	16	6/16[g]	37.5
Tumor Homogenates[c]	6	6	1/6	17.0
SQ Injection[d]	44	23	0/23	0
IP Injection[d]	6	1	0/1	0
ID Injection[d]	14	13	11/13	85.0
Controls[e]	140	0	-	-

[a] All animals initially challenged with MCA-1 tumor tissue were observed for evidence of tumor growth for 16 weeks. The number of animals initially challenged with MCA-1 and the number of animals surviving the tumor challenge (i.e., no tumor growth) were recorded.

[b] Normal animals were inoculated with two 1-2 mm pieces of MCA-1 in the rear flank with a trocar piece.

[c] Normal animals were injected SQ with 1.0 ml of a tumor homogenate, prepared in saline (50% v/v).

[d] Normal animals were injected subcutaneously (SQ), intraperitoneally (IP) or intradermally (ID) with 1.0 ml of a fine tumor mince of MCA-1, prepared in saline (50% v/v).

[e] Normal animals were injected subcutaneously in the rear flank with 1.0 ml of a single cell suspension (SCS) of MCA-1 containing 1×10^8 cells (85-95% viability). All control animals died within 5 weeks after challenge.

[f] All survivors were rechallenged with a SCS of MCA-1 (1×10^8 cells) in the rear flank and observed for 16 weeks for evidence of tumor growth. The number (and percent) of animals surviving rechallenge with SCS were recorded. No tumor growth was observed in surviving animals. The survivors of a SCS rechallenge were considered to be immune to MCA-1.

[g] $\frac{\text{Number of animals surviving SQ rechallenge with SCS}}{\text{Number of animals surviving initial tumor challenge}}$

Table 3

Delayed Skin Test Response to TSA (KCl-Extracted Tumor Antigens) in Normal, Immune and Tumor-Bearing Strain 13 Guinea Pigs

Group	Number of Animals	Delayed Skin Test[c] (mm induration ± S.D.)
Normal	8	1.6 ± 2.5
Immune[a]	44	7.7 ± 4.3[d]
Tumor-bearing[b]	3	3.7 ± 1.2

[a] All immune animals were rechallenged subcutaneously with SCS (1×10^8 cells) to confirm tumor immunity 16 weeks prior to skin testing.

[b] All tumor-bearing animals had tumors ranging from 3 to 5 cm in size.

[c] All animals were injected intradermally with 0.1 ml of a single preparation of TSA (KCl-extracted tumor antigens, 20 mg protein/ml antigens). Delayed skin reactions (mm induration ± S.D.) were measured and recorded 24 hours later.

[d] $P < 0.05$ when immune and control groups are compared. (Students t test)

TABLE IV

DELAYED SKIN TEST REACTIVITY OF KCL-EXTRACTED MCA–1 TUMOR ANTIGENS ISOLATED BY DISC ELECTROPHORESIS IN STRAIN 13 GUINEA PIGS[a]

Group	No. Animals	Fraction 1	2	3	4	5	6	7
Immune	7	-C	-	±	+	±	-	-
Control	2	-	-	-	-	-	-	-

[a]MCA 1 immune or control strain 13 guinea pigs were skin tested with seven saline eluates of KC1-extracted tumor antigen after separation by disc electrophoresis.

[b]The tumor antigen sample (0.84 mg protein) was electrophoresed for 45 minutes at 1.5 mA/gel. Stained and unstained gels were compared to arbitrarily divide the test gel into the seven fractions indicated.

[c]– no visible reaction
+ > 3 mm induration or erythema in all animals

± > 3 mm induration or erythema in some animals

Table 5

In Vitro Lymphocyte Transformation Reactivity of MCA-1 Immune or Tumor-Bearing Strain 13 Guinea Pigs to TSA[a]

		Stimulation Index[b]				
Group	No. Animals	<1	1-2	2-3	3-4	4-5
Immune	26	6	13	5	1	1
Tumor-Bearing	4	0	1	1	2	0
Control	12	2	6	3	1	0

[a] Lymphocyte responsiveness to TSA (20 mg/protein/ml; 0.1 ml/tube) using the whole blood culture system described in Methods. Cultures were incubated for 6 days at 37 C.

[b] Counts per minute (CPM) of tritiated thymidine incorporation after a 24 hour pulse using 1.0 μCi/tube.

$$\text{Stimulation index} = \frac{\text{mean CPM test}}{\text{mean CPM control}}$$

Controls were between 400-1500 CPM.

Table 6

Serum Globulin Changes in Tumor-Bearing, Immune and Control Guinea Pigs

Prospective Groups	Globulin Fraction									
	1	2	3	4	5	6	7	8	9	10
Normal	16.3[b] ± 1.3	24.0 ± 1.8	3.5 ±0.43	14.4 ± 0.92	7.0 ±0.68	8.8 ±0.70	5.8 ±0.72	11.0 ± 0.9	4.9 ±0.5	3.8 ±0.45
Tumor-Bearing	14.7 ± 1.8	23.5 ± 1.5	4.6 ±0.7	18.9[c] ± 1.5	6.0 ±0.5	6.4 ±1.4	5.4 ±0.5	10.8 ± 1.3	5.5 ±0.7	3.7 ±0.8
Immune	14.7 ± 1.4	23.0 ± 2.3	5.2 ±0.4	13.6 ± 1.3	7.2 ±0.2	9.5 ±0.4	7.2 ±1.2	10.6 ± 1.0	4.9 ±0.5	3.7 ±1.0
Retrospective[d] Group										
Tumor-Bearing	13.9 ± 1.6	23.6 ± 2.8	6.4 ±1.8	19.1[e] ± 2.2	5.4 ±1.0	8.1 ±2.6	3.5 ±1.2	9.4 ± 2.0	6.5 ±1.4	4.2 ±0.9

[a] Serum from Strain 13 guinea pigs (5 animals/group) was analyzed by disc gel electrophoresis. The mean globulin percent of 10 globulin fractions was determined.

[b] Mean globulin percent of 10 globulin fractions ± standard deviation.

Table 6, continued

[c] Significant elevation of fraction 4 in tumor-bearing animals compared to both immune or control animals ($P < 0.01$). (Students t test)

[d] Twenty-eight frozen serum samples from tumor-bearing animals were analyzed retrospectively as above.

[e] Significant elevation of fraction 4 in frozen serum samples compared to immune or control animals ($P < 0.01$). (Students t test)

Table 7

Levels of Fraction 4 in Tumor-Challenged and Control Strain 13 Guinea Pigs[a]

Expt.	Animal	Time After Tumor Challenge (Days) 0	4	8	12	16	20	24	28	32
Tumor Group										
I	1	17.6[b]	17.2	15.0	19.3	24.4	21.3	21.7		
	2	16.1	16.6	16.6	15.2	21.5	21.7	-[c]		
	3	17.1	17.8	15.8	17.1	15.3	15.2	26.2		
	4	16.1	16.6	15.6	20.9	21.7	21.1	-		
	5	13.9	16.0	18.3	14.3	18.9	23.0	-		
II	1	ND[d]	14.9	15.1	16.1	18.2	21.8	27.3	22.4	21.6
	2	ND	13.8	15.8	16.5	16.9	21.6	25.3	20.1	15.2
	3	ND	17.7	16.7	17.8	16.3	25.9	29.7	24.9	20.7
	4	ND	15.7	13.1	14.3	16.1	16.6	23.4	21.8	18.9
	5	ND	14.0	14.7	15.4	15.6	14.7	19.3	26.2	23.3
	6	ND	15.0	15.0	14.5	18.8	28.5	24.3	23.6	10.5
Mean ± SD		16.2 ± 1.4	15.9 ± 1.4	15.6 ± 1.3	16.5 ± 2.1	18.5 ± 2.9	21.0 ± 4.2	24.7 ± 3.3	23.2 ± 2.2	20.0 ± 2.8
Control Group										
I	1	16.3	17.6	17.8	14.5	16.3	18.5	-		
	2	16.9	16.0	16.3	13.9	15.2	14.9	-		
	3	15.8	17.8	18.0	15.5	16.3	15.6	-		

II	1	ND	15.2	14.3	14.8	15.9	14.9	14.1	11.3	14.3
	2	ND	14.3	16.5	15.3	14.9	16.6	16.1	14.5	13.5
Mean ± SD		16.3 ± 0.6	16.2 ± 1.5	16.6 ± 1.4	14.8 ± 0.6	15.7 ± 0.6	16.1 ± 1.5	15.1 ± 1.4	12.9 ± 2.3	13.9 ± 0.6
P <		0.5[e]	0.5	0.2	0.1	0.05	0.05	0.01	0.01	0.02

[a] Serum from Strain 13 guinea pigs was analyzed by disc gel electrophoresis. The mean globulin percent of ten globulin fractions was calculated after densitometry. The fourth globulin fraction increases during tumor growth compared to controls.

[b] Fraction 4, percent of total globulin (mean ± SD).

[c] Animals sacrificed.

[d] ND, not done.

[e] Significance of comparisons between mean fraction 4 levels of tumor challenged to control animals. (Students t test)

Table 8

Tumor Growth in Strain 13 Guinea Pigs After Subcutaneous Challenge with MCA-1 Tumor[a]

		Time After Tumor Challenge (Days)								
Expt.	Animal	0	4	8	12	16	20	24	28	32
I	1	0[b]	0	0.5	1.5	2.0	8.0	8.0[c]		
	2	0	0	0	1.0	2.0	8.0	8.0		
	3	0	0	0	0	0	0	8.0		
	4	0	0	0	0	1.5	8.0	8.0		
	5	0	0	0	1.0	2.0	3.0	7.0		
II	1	0	0	0	0	0	4.0	5.0	6.0	7.5
	2	0	0	0	0	1.5	4.0	5.0	5.5	5.5
	3	0	0	0	0.5	1.0	3.5	5.0	6.0	6.5
	4	0	0	0	0	1.0	2.0	3.0	3.0	3.0
	5	0	0	0	0	0	4.0	4.0	6.0	7.0
	6	0	0	0	0.5	1.0	4.0	5.5	6.5	7.0
Mean[d]		0	0	0.5	0.9	1.5	4.35	5.85	5.5	6.1

[a] Animals were inoculated subcutaneously with 1.0 ml of a single cell suspension of MCA-1 in the rear flank (1×10^8 cells).

[b] Average tumor diameter from two perpendicular diameters (cm).

Table 8, continued

[c] Animals sacrificed after tumor measurement.

[d] Mean tumor size (cm) of animals bearing tumors.

Table 9

Serum Globulin Changes in Hartley Guinea Pigs During Sensitization to 2,4-Dinitrofluorobenzene (DNFB)[a]

Electrophoretic fraction[b]	Day of Bleeding[c] -1	3	6	10	11	17
1	19.9 ± 6.1[d]	18.8 ± 3.6	17.9 ± 3.0	20.0 ± 3.0	22.1 ± 3.0	20.8 ± 3.7
2	23.6 ± 4.4	20.4 ± 3.1	20.5 ± 2.8	19.8 ± 2.3	21.0 ± 2.8	22.1 ± 3.1
3	3.0 ± 0.7	3.5 ± 0.5	3.5 ± 0.5	3.4 ± 1.1	3.0 ± 1.1	3.4 ± 0.7
4	10.2 ± 1.6	17.0 ± 3.4[e]	17.1 ± 2.6[f]	13.1 ± 2.1	11.9 ± 1.6	9.6 ± 1.0
5	5.3 ± 1.1	4.7 ± 1.1	4.6 ± 1.1	5.3 ± 1.7	5.0 ± 1.1	5.3 ± 1.5
6	11.9 ± 2.9	12.2 ± 2.3	12.6 ± 2.6	13.4 ± 2.7	12.7 ± 2.7	12.5 ± 2.0
7	12.5 ± 5.9	10.6 ± 4.7	10.4 ± 4.6	10.9 ± 3.7	11.0 ± 4.1	12.0 ± 3.9
8	7.7 ± 1.5	7.2 ± 1.9	7.0 ± 2.0	7.8 ± 2.0	7.2 ± 2.0	8.1 ± 2.3
9	5.8 ± 1.2	5.7 ± 1.3	6.3 ± 1.6	6.3 ± 1.4	6.0 ± 0.9	6.2 ± 2.0

Table 9, continued

[a] Animals were sensitized to DNFB for 6 consecutive days (days 1-6).

[b] Each fraction may contain more than one electrophoretic species.

[c] Animals were bled on days -1, 3, 6, 10, 11, and 17.

[d] Mean globulin percent ± S.D. of each fraction determined from a group of ten guinea pigs.

[e] $P < 0.01$; compared to fraction 4, DNFB-group, bleeding day -1 and control group, bleeding day 3. (Students t test)

[f] $P < 0.01$; compared to fraction 4, DNFB-group, bleeding day -1 and control group, bleeding day 6. (Students t test)

Table 10

Serum Globulin Levels in Control Group Guinea Pigs

Electrophoretic fraction[a]	Day of Bleeding[b] -1	3	6	10	11	17
1	20.7 ± 3.1[c]	20.5 ± 2.2	20.6 ± 1.6	20.4 ± 1.9	20.8 ± 1.7	20.7 ± 1.7
2	22.1 ± 2.6	21.8 ± 1.7	21.2 ± 1.6	22.4 ± 1.9	21.3 ± 1.6	19.8 ± 3.4
3	2.6 ± 0.6	3.0 ± 0.3	2.8 ± 0.4	2.7 ± 0.8	3.2 ± 0.3	3.4 ± 0.4
4	9.8 ± 1.1	10.0 ± 1.1	10.3 ± 1.3	10.0 ± 0.6	9.7 ± 0.9	9.8 ± 0.8
5	5.9 ± 0.8	6.2 ± 0.9	5.9 ± 0.9	5.4 ± 0.9	5.9 ± 1.0	6.0 ± 0.9
6	11.0 ± 2.2	11.0 ± 2.1	11.2 ± 2.3	11.5 ± 2.3	11.4 ± 2.0	12.0 ± 2.1
7	13.1 ± 2.8	12.5 ± 2.9	13.0 ± 1.8	12.8 ± 2.4	12.6 ± 2.9	13.4 ± 2.0
8	7.6 ± 1.8	7.6 ± 1.9	7.2 ± 2.4	7.1 ± 1.9	7.6 ± 1.6	7.3 ± 1.6
9	7.1 ± 1.3	7.2 ± 1.0	7.5 ± 1.0	7.4 ± 1.3	7.5 ± 1.3	7.0 ± 1.3

[a] Each fraction may contain more than one electrophoretic species.

[b] Animals were bled on days -1, 3, 6, 10, 11, and 17.

[c] Mean globulin percent ± S.D. of each fraction determined from a group of ten guinea pigs.

Table 11

Changes in Serum Globulin Fraction 4 During Sensitization

To *Mycobacterium Tuberculosis* H37RA

Guinea pig	Adjuvant control group	Immunized group			
		Postimmunization			
	Highest values	Preimmunization	Highest value	Bleeding	Percent increase
1	10.1[a]	9.5	17.8	6	87
2	9.2	9.6	14.0	4	46
3	8.8	9.3	18.7	2	101
4	11.1	6.5	17.8	4	174
5	9.1	8.9	13.1	3	47
6	9.6	10.2	10.2	1	0
7	9.8	7.1	13.5	6	90
8	10.3	9.2	12.5	2	36
9	10.2	9.3	13.3	3	43
10	12.2	12.5	15.5	4	24
11	9.1	11.8	17.0	3	44
12	8.6	8.0	12.7	2	59
13	8.7	8.1	11.1	3	37
Mean ± SD	9.8 ± 1.9	9.2 ± 1.7	14.4 ± 2.7[b]		

[a] Percent fraction 4 of total globulin.

[b] $P < 0.01$; compared to adjuvant alone or preimmunization values. (Students t test)

Table 12

Lymphocyte Responsiveness to PHA Stimulation in Tumor-Challenged and Control Strain 13 Guinea Pigs[a]

Expt.	Animal	Time After Tumor Challenge (Days) 0	4	8	12	16	20	24	28	32
Tumor Group										
I	1	25.0[b]	0.5	33.0	5.0	1.6	4.0	[c]		
	2	54.0	20.0	60.0	10.0	10.5	9.0			
	3	36.0	4.5	45.0	35.0	2.8	13.0			
	4	45.0	52.0	14.0	8.0	4.4	1.9			
	5	27.0	60.0	55.0	50.0	18.5	13.0			
Control Group										
	1	25.0	20.0	45.0	8.0	8.0	22.5			
	2	13.0	ND[d]	18.0	12.0	14.0	26.0			
	3	55.0	23.0	60.0	45.0	10.0	31.0			
Tumor Group										
II	1	ND	32.0	38.0	33.0	14.0	25.0	18.0	18.0	4.7
	2	ND	54.0	58.0	19.5	23.0	23.0	12.0	2.4	4.0
	3	ND	29.0	58.0	7.5	11.0	14.0	5.0	2.9	1.1
	4	ND	24.0	36.0	22.0	35.0	23.0	15.0	14.0	0.9
	5	ND	15.0	11.0	5.5	6.0	9.5	8.0	7.9	4.0
	6	ND	25.0	72.0	38.0	31.0	8.0	3.5	2.9	3.0

Control Group

1	ND	13.0	52.0	21.0	25.0	54.0	37.0	35.0	17.0
2	ND	13.0	22.0	44.0	48.0	48.0	50.0	59.0	42.0

[a] Lymphocyte responsiveness to PHA stimulation using the whole blood culture system described in Methods. Cultures were incubated for 6 days at 37 C with 10 µg PHA-P.

[b] Counts per minute (CPM) x 10^3 of tritiated thymidine incorporation after a 24 hr pulse using 1.0 µCi/tube.

[c] Animals sacrificed.

[d] ND, not done.

Table 13

Lymphocyte Responsiveness to PWM Stimulation in Tumor-Challenged and Control Strain 13 Guinea Pigs[a]

Expt.	Animal	Time After Tumor Challenge (Days) 0	4	8	12	16	20	24	28	32
Tumor Group										
I	1	50.0[b]	14.0	18.0	16.0	0.6	5.2	[c]		
	2	60.0	45.0	70.0	35.0	20.0	9.0			
	3	85.0	45.0	58.0	76.0	0.4	40.0			
	4	85.0	42.0	35.0	100.0	3.9	0.9			
	5	170.0	50.0	80.0	110.0	70.0	13.0			
Control Group										
	1	60.0	52.0	43.0	62.0	35.0	42.0			
	2	50.0	ND[d]	50.0	65.0	58.0	47.0			
	3	75.0	11.0	50.0	55.0	30.0	67.0			
Tumor Group										
II	1	ND	11.5	68.0	74.0	47.0	50.0	4.0	1.2	0.5
	2	ND	32.0	80.0	68.0	60.0	41.0	4.8	0.4	3.4
	3	ND	42.0	140.0	87.0	44.0	20.0	1.5	0.2	0.1
	4	ND	22.5	74.0	55.0	37.0	8.0	21.0	0.2	0.1
	5	ND	50.0	118.0	11.0	46.0	118.0	5.5	3.5	4.0
	6	ND	25.0	87.0	57.0	70.0	4.5	0.2	0.6	0.3

Control Group									
	1	ND	35.0	73.0	82.0	58.0	48.0	26.0	77.0	43.0
	2	ND	40.0	163.0	75.0	55.0	60.0	80.0	69.0	39.0

[a] Lymphocyte responsiveness to PWM stimulation using the whole blood culture system described in Methods. Cultures were incubated for 6 days at 37 C with 0.1 ml of a 1/10 dilution of PWM.

[b] Counts per minute (CPM) x 10^3 of tritiated thymidine incorporation after a 24 hr pulse using 1.0 µCi/tube.

[c] Animals sacrificed.

[d] ND, not done.

The Effects of Tumor-Bearing or Normal Strain 13 Serum on the Delayed Skin Test Reactivity of Dinitrofluorobenzene and Tuberculin Sensitive Guinea Pigs

Group	Treatment[a]	Response before Treatment[c]	Response after Treatment[d]
DNFB[c]	Saline	5/5	5/5
PPD[d]		5/5	5/5
DNFB	Tumor-bearing serum	5/5	1/5
PPD		5/5	1/5
DNFB	Normal serum	2/2	1/2
PPD		2/2	2/2

[a] Animals were injected intraperitoneally with 5.0 ml of serum or saline for three consecutive days.

[b] The fraction of animals showing normal delayed skin test reactivity/ number of animals tested.

[c] DNFB-sensitive guinea pigs were skin tested one month prior to and on the last day of serum treatment with DNFB. Reactions were observed 24 and 48 hours later. A positive response was recorded if there was homogenous erythema at the skin test site.

[d] Tuberculin sensitive guinea pigs were skin tested one month prior to and on the last day of serum treatment with 5.0 µg PPD. Reactions were observed 24 and 48 hours later. A positive response was

recorded if the skin test site in treated animals was greater than 15 mm of induration (10 control animals measured 18-25 mm induration).

Table 15

The Effects of Tumor-Bearing or Normal Strain 13 Serum on Tumor Growth in Outbred Hartley Strain Guinea Pigs

Group	No. Animals	Treatment[a]	Number of Animals with Palpable Tumors (DAYS AFTER TUMOR CHALLENGE) 7	14	21	28	35
Hartley	6[b]	Normal Strain 13 serum	2	2	0	0	0
Hartley	6	Tumor-bearing Strain 13 serum	3	5	3	0	0
Hartley	20	-	0	1	1	1	0
Strain 13	8[c]	-	8	8	8	-[d]	-

[a] Outbred Hartley guinea pigs (age and sex matched) were injected intraperitoneally with 5.0 ml of Strain 13 normal or tumor-bearing serum for three consecutive days following tumor challenge, or were untreated.

[b] All Hartley guinea pigs were challenged subcutaneously (rear flanks) and intraperitoneally with a single cell suspension of MCA-1 (1.0 ml/site, 1 x 10^8 cells) and palpated at 3 or 4

Table 15, continued

day intervals for evidence of tumor growth.

[b] Inbred Strain 13 control guinea pigs were injected in the rear flanks only (1.0 ml/site) with SCS (1×10^8 cells).

[c] All Strain 13 control animals died from the tumor by day 28.

Table 16

The Effects of Dinitrofluorobenzene Sensitization on

Phytohemagglutinin Induced Lymphocyte Transformation

Treatment Schedule[b]		Lymphocyte Transformation[a]			
		Experiment - 1		Experiment - 2	
		No. Animals	CPM	No. Animals	CPM
Before Sensitization (Day-1)		36	34,000 ± 4,000	32	52,000 ± 8,000
During Sensitization (Day+5)					
	Control	18	38,000 ± 4,600	16	48,000 ± 7,200
	DNFB	18	36,800 ± 1,800 (80%)[c]	16	18,700 ± 5,600 (64%)
Post Sensitization (Day+12)					
	Control	17	39,000 ± 4,900	16	55,000 ± 9,000
	DNFB	18	21,600 ± 4,000 (27%)	15	42,300 ± 4,300 (19%)

[a] Whole blood cultures were stimulated with 10 µg PHA. CPM = counts per minute of the mean ± standard error of triplicate cultures from the indicated number of animals. All cultures were pulsed for 24 hr with 1 µCi ^{3}H-thymidine before harvesting. Controls without PHA were always less than 1,000 CPM (600 ± 200 for all experiments).

Table 16, continued

[b] In each experiment, animals were cultured prior to treatment and divided into two groups. One group (- control) was treated with ethanol and the other group (- DNFB) was treated with 2% DNFB in ethanol. All animals were cultured on the fifth day of treatment and 7 days after the termination of treatment.

[c] Percent suppression as compared to pretreatment values.

Table 17

The Effects of Dinitrofluorobenzene Sensitization on

Pokeweed Mitogen Induced Lymphocyte Transformation

Treatment Schedule[b]		Lymphocyte Transformation[a]			
		Experiment - 1		Experiment - 2	
		No. Animals	CPM	No. Animals	CPM
Before Sensitization (Day-1)		36	46,000 ± 4,000	32	71,000 ± 5,000
During Sensitization (Day+5)					
	Control	18	42,000 ± 6,000	16	76,000 ± 6,000
	DNFB	18	7,800 ± 1,700 (83%[c])	16	18,500 ± 4,400 (74%)
Post Sensitization (Day+12)					
	Control	17	45,000 ± 3,000	16	68,000 ± 6,000
	DNFB	18	37,000 ± 7,000 (20%)	15	58,000 ± 6,000 (18%)

[a] Whole blood cultures were stimulated with 0.1 ml of 1/10 PWM. CPM = counts per minute of the mean ± standard error of triplicate cultures from the indicated number of animals. All cultures

Table 17, continued

were pulsed for 25 hr with 1 μCi ^{3}H-thymidine before harvesting. Controls without PWM were always less than 1,000 CPM (600 ± 200 for all experiments).

[b] In each experiment animals were cultured prior to treatment and divided into two groups. One group (- control) was treated with ethanol and the other group (- DNFB) was treated with 2% DNFB in ethanol. All animals were cultured on the fifth day of treatment and 7 days after the termination of treatment.

[c] Percent suppression as compared to pretreatment values.

Table 18

The Effects of Dinitrofluorobenzene Sensitization on Concanavalin A Induced Lymphocyte Transformation

Treatment Schedule[b]		Lymphocyte Transformation[a] No. Animals	CPM
Before Sensitization (Day-1)		32	26,000 ± 3,000
During Sensitization (Day+5)			
	Control	16	27,000 ± 4,200
	DNFB	16	11,000 ± 2,400 (58%)[c]
Post Sensitization (Day+12)			
	Control	16	20,800 ± 3,000
	DNFB	15	17,000 ± 3,100 (34%)

[a] Whole blood cultures were stimulated with 5 µg Con A. CPM = counts per minute of the mean ± standard error of triplicate cultures from the indicated number of animals. All cultures were pulsed for 24 hr with 1 µCi ^{3}H-thymidine before harvesting. Controls without Con A were always less than 1,000 CPM (600 ± 200 for all experiments).

[b] In each experiment animals were cultured prior to treatment and divided into two groups. One group (- control) was treated with ethanol and the other group (- DNFB) was treated with 2% DNFB in ethanol. All animals were cultured on the fifth day of treatment and 7 days after the termination of treatment.

[c] Percent suppression as compared to pretreatment values.

Table 19

The Effects of Dinitrofluorobenzene Sensitization on

Tuberculin Sensitive Guinea Pigs Measured by Lymphocyte Transformation

Treatment Schedule[a]		No. Animals	Lymphocyte[b] Transformation (CPM)	Level of F4[c] (Percent)
Before Sensitization (Day-1)		32	21,300 ± 3,800	14.0 ± 1.6
During Sensitization (Day-5)				
	Control	16	19,800 ± 4,200	13.8 ± 1.8
	DNFB	16	4,800 ± 1,200 (77%)[d]	20.6 ± 2.1
Post Sensitization (Day-12)				
	Control	16	22,000 ± 4,600	14.2 ± 1.8
	DNFB	15	18,000 ± 3,600 (16%)	15.8 ± 2.4

[a] All animals were sensitized to tuberculin 45 days prior to these manipulations. In each experiment animals were cultured prior to treatment and divided into two groups. One group (- control) was treated with ethanol and the other group (- DNFB) was treated with 2% DNFB

Table 19, continued

in ethanol. All animals were cultured on the fifth day of treatment, and 7 days after the termination of treatment.

[b] Whole blood cultures were stimulated with 5 µg tuberculin (PPD). CPM = counts per minute of the mean ± standard error of triplicate cultures from the indicated number of animals. All cultures were pulsed for 24 hr with 1 µCi ^{3}H-thymidine before harvesting. Controls without PPD were always less than 1,000 CPM (600 ± 200 for all experiments).

[c] Levels of F4 were determined by disc gel electrophoresis of serum (8-12 λ, approximately 0.7 mg protein) and expressed as mean ± S.D. of the total globulin.

[d] Percent suppression as compared to pretreatment values.

Table 20

The Effects of Dinitrofluorobenzene Sensitization on Tuberculin Sensitive Guinea Pigs Measured *In Vivo*[a]

Animal No.	Delayed Dermal Response to 5 μg Tuberculin PPD (mm induration)		
	Before DNFB Sensitization (Day-1)	During DNFB Sensitization (Day+5)	Post DNFB Sensitization (Day+12)
1	22 (N)[b]	14	19 (N)
2	20 (N)	17	22 (N)
3	19	15	20
4	21 (N)	15	21
5	24 (N)	15	21 (N)
6	21 (N)	14	20
7	20 (N)	12	19 (N)
8	ND	17	22 (N)
9	ND	15	19
10	ND	14	17 (N)
11	ND	12	20 (N)
12	ND	13	16
13	ND	14	23 (N)
14	ND	9	15
15	ND	15	20 (N)
F4 globulin[c] (Percent)	14.0 ± 1.5	20.6 ± 2.1	15.8 ± 2.4

[a] All animals were sensitized to tuberculin 45 days prior to this experiment. The tuberculin response was measured *in vivo* with a

Table 20, continued

dermal skin test to 5 μg PPD. The reactions were graded at 24 hours according to diameter of induration (mm) and intensity (central necrosis).

[b] (N) large (5 mm) central necrosis was noted. ND not done.

[c] Levels of F4 were determined by disc gel e.ectrophoresis of serum (8-12 λ, approximately 0.7 mg protein) and expressed as mean ± S.D. of the total globulin.

Table 21

Assay of Immune Reactivity in TF Donor Animals[a]

TF donor group	Animal	Delayed skin test response (mm induration)	MI (+ = >50% inhibition)
Immune	1	15	+
	2	5	+
	3	11	-
	4	12	+
	5	7	-
	6	9	+
	7	7	ND
	8	7	ND
	9	5	+
	10	4	ND
	11	0	+
	12	8	+
	13	3	+
	14	0	+
Control	1	0	-
	2	0	-
	3	2	-
	4	0	-

[a] Delayed skin test reactivity and the degree of migration inhibition to TSA were assessed in all animals prior to transfer as described in Methods.

Table 22

Assay of Immune Reactivity in TF Recipient Animals[a]

TF recipient group	Animal	Delayed skin test response (mm induration)	Tumor growth 10 days after MCA-1 challenge [b]	Metastases on day 20[c]
Immune	1	4	+	+
	2	0	+	-
	3	0	+	-
	4	5	+	-
	5	3	-	+
	6	5	+	-
	7	4	-	+
	8	2	-	-
	9	3	+	-
	10	Dead	+	-
	11	0	+	-
	12	2	-	-
	13	2	+	-
	14	2	+	-
Control	1	2	+	-
	2	3	+	-
	3	2	+	-
	4	2	+	-

[a] Delayed skin test reactivity was assessed with TSA as described in Methods. Tumor resistance and the occurrence of metastatic growth were assessed after challenge with viable MCA-1 tumor tissue.

[b] + Presence of palpable tumor (approximately 1.0 cm).

- Absence of palpable tumor.

Table 22, continued

[c] + Metastatic tumors remote from the injection site.

- No apparent metastases

Table 23

Summary of Tumor Growth in Recipients of Transfer Factor From MCA-1 Immune Donors

Recipient Group	No. Animals	Day of observation[a] 10 Tumor Growth[b]	15 Tumor size[c]	20 Animals with Metastases[d]	34 Survivors
Immune TF	14	10/14	9/14 3.0 cm	3/14	0/14
Control TF	4	4/ 4	4/4 3.0 cm	0/ 4	0/ 4

[a] During the 34 day observation period, early tumor acceptance (day 10), tumor growth rate (day 15), metastatic spread (day 20), and number of survivors (day 34) were recorded.

[b] The number of immune or control group animals with palpable tumors/number of recipients.

[c] The number of immune or control group animals bearing tumors less than 3.0 cm in diameter on day 15/number of recipients of TF.

[d] The number of immune or control group animals with metastatic tumors remote from the injection site on day 20/number of recipients of TF.

[e] The number of immune or control group animals surviving MCA-1 challenge on day 34/number of recipients of TF.

Figure 1

FLOW CHART OF EXTRACTION, PARTIAL PURIFICATION AND CONCENTRATION OF SOLUBLE TUMOR ANTIGENS

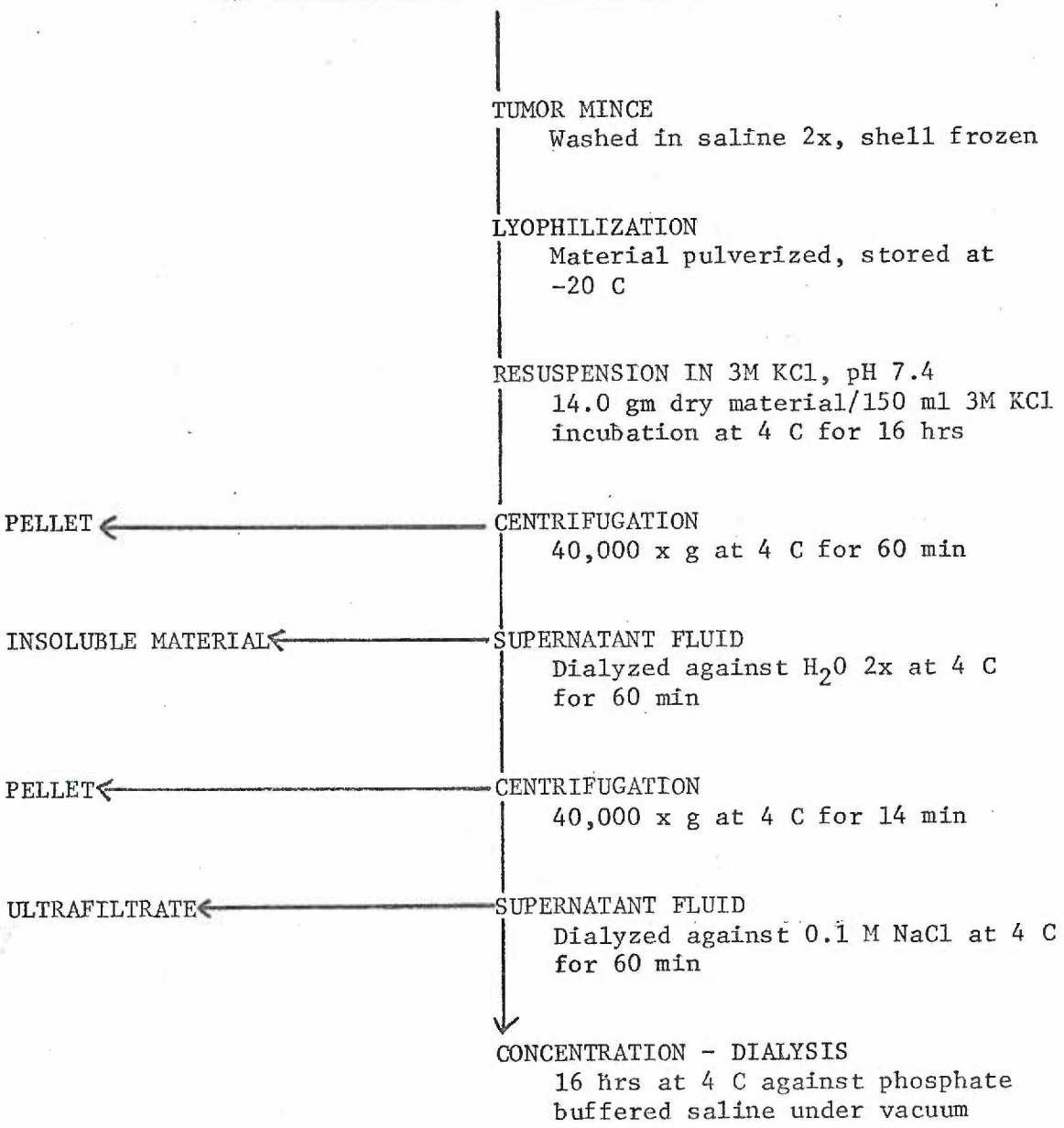


Figure 2 .

A comparison of average growth rates of the first passage of DMBA (-●-●-), BP (-□-□-) and MCA (-▲-▲-) induced tumors in Strain 13 guinea pigs. Twenty-five animals per group were injected with 4, 1-2 mm pieces of tumor tissue by trocar inoculation. Each curve represents the average tumor growth rate in 20, 21 and 20 tumor-bearing animals from the DMBA, BP and MCA groups respectively. Tumor size was estimated by averaging perpendicular diameters at 4-day intervals until death. Variation in tumor size was less than 1.0 cm on any one day within each group.

GROWTH RATE OF TUMORS IN VIVO

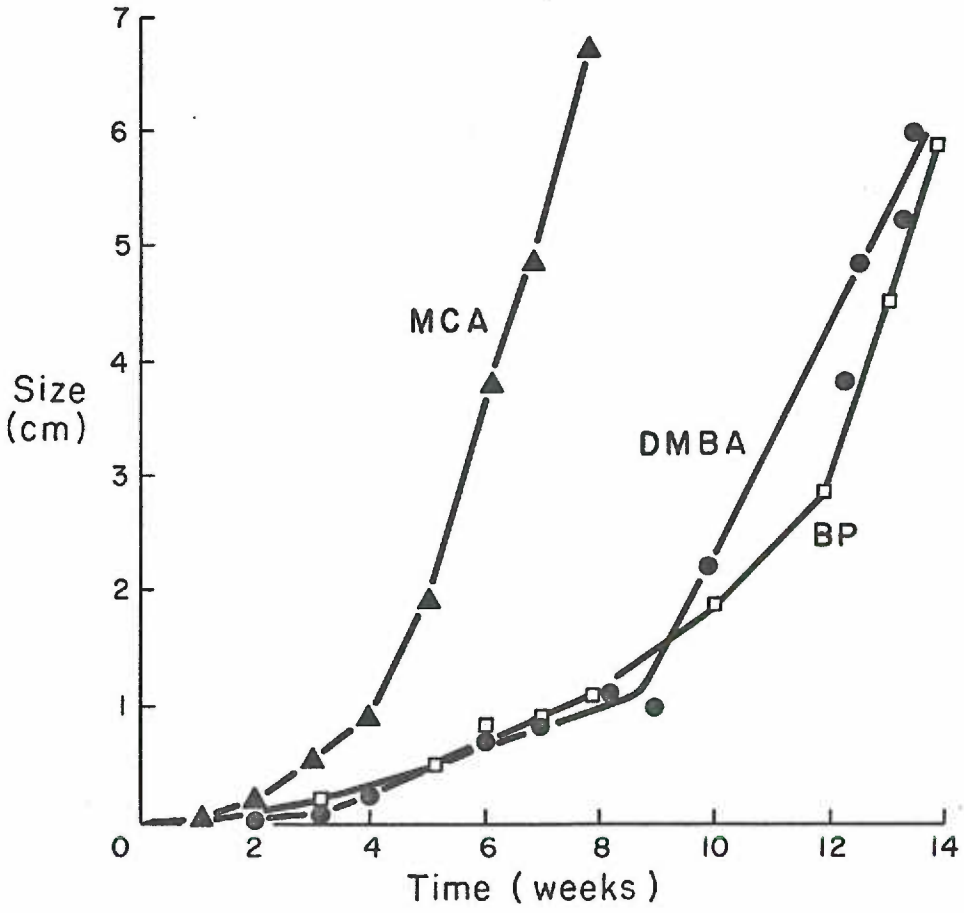


Figure 3

A comparison of growth rates of MCA-1 tumor tissue (passage 8) injected by trocar (—Δ-Δ-) or as a single cell suspension (1 x 10^8 cells) (-●-●-) in 20 and 11 Strain 13 guinea pigs respectively. Tumor size was estimated by averaging perpendicular diameters at 4-day intervals until death. Variation in tumor size was less than 1.0 cm on any one day within each group.

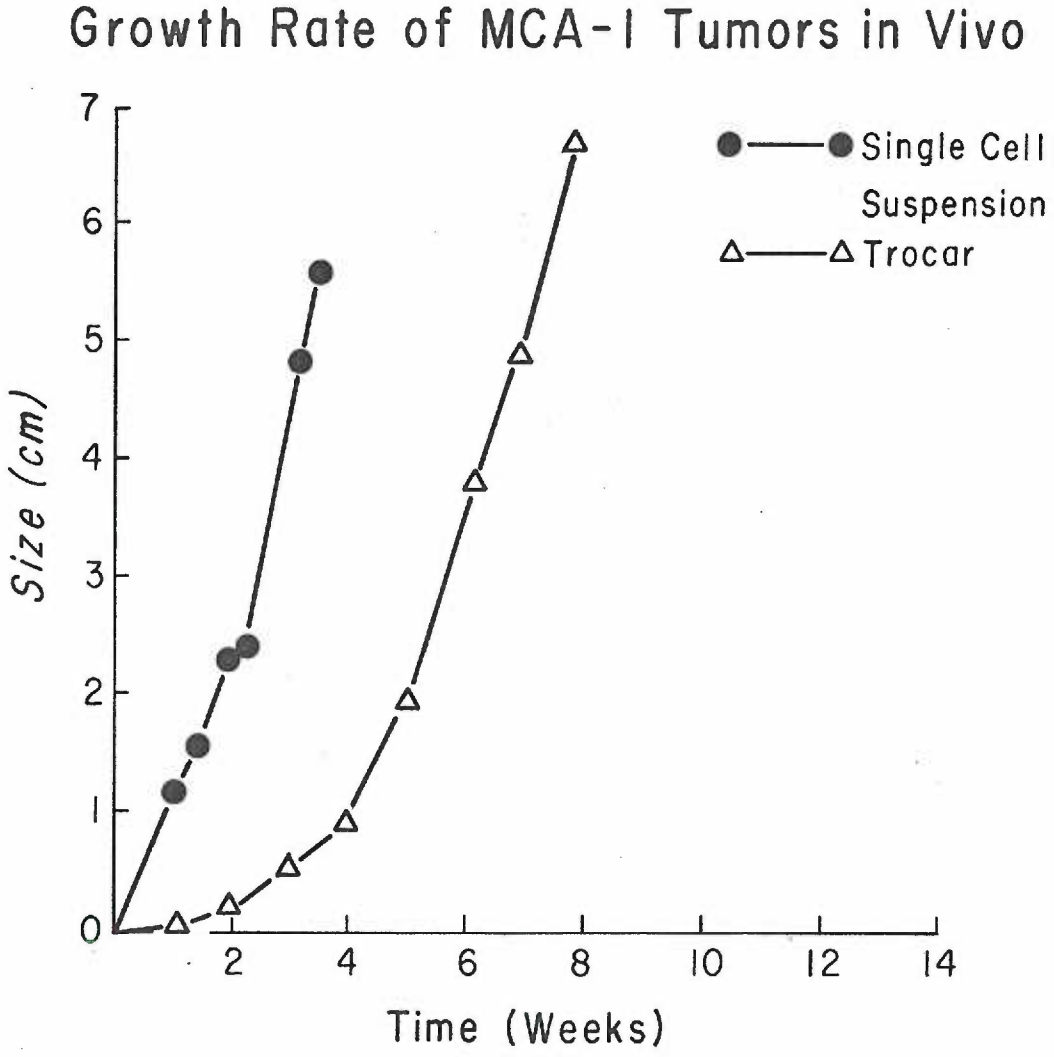

Growth Rate of MCA-1 Tumors in Vivo
Size (cm)
0
1
2
3
4
5
6
7
2
4
6
8
10
12
14
Time (Weeks)
Single Cell Suspension
Trocar

Figure 4

Strain 13 guinea pig bearing MCA-1-induced tumors in each rear flank after injection with SCS 4 weeks earlier. Each tumor is approximately 6.0 cm in diameter.

Figure 5

Histological section of MCA-1 tumor tissue stained with Hematoxalin and Eosin. Well-defined tumor cells containing spindle-shaped nuclei are noted (500X).

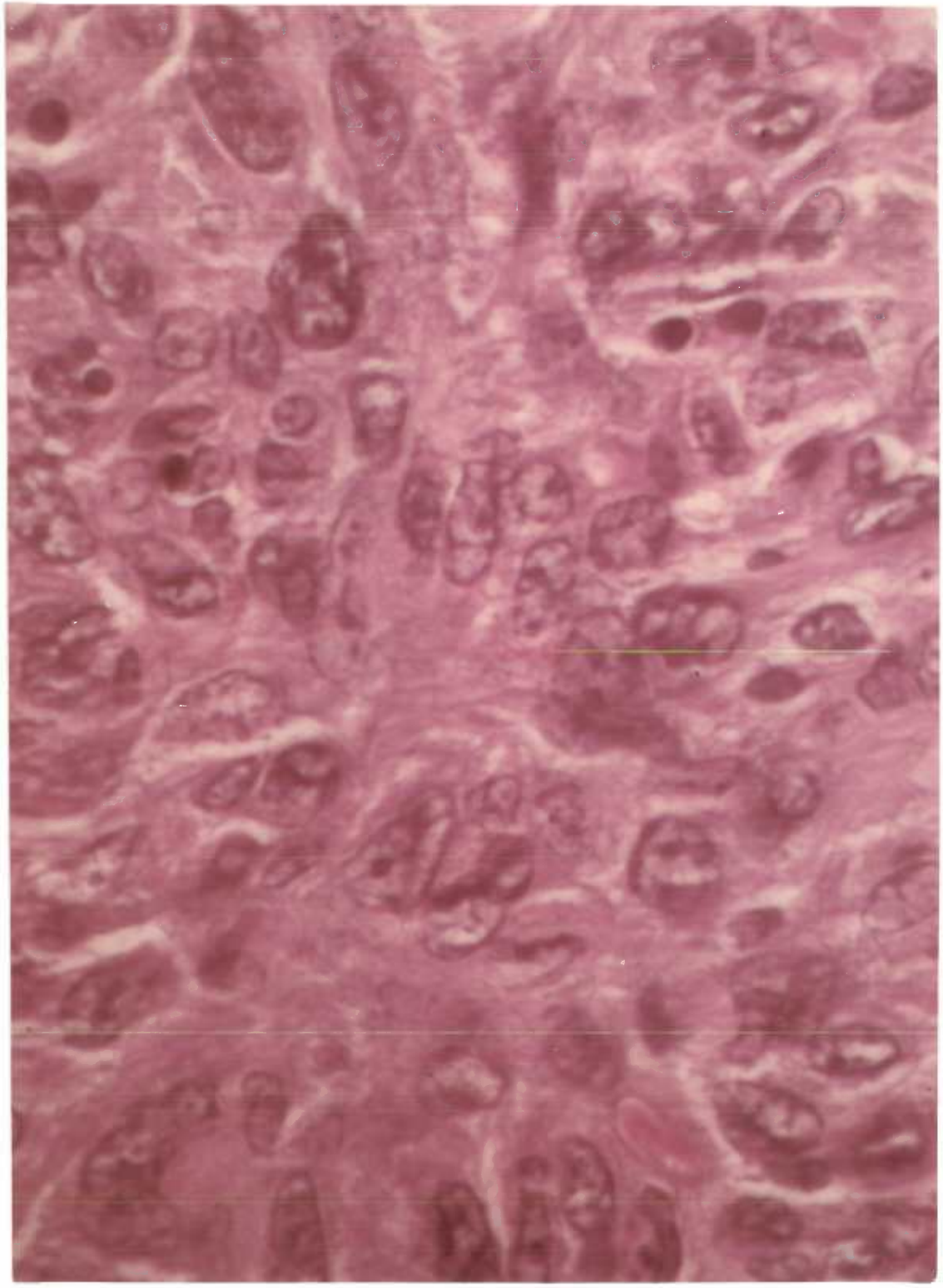


Figure 6

Histological section of MCA-1 tumor tissue. Large spindle-shaped tumor cells containing dense granules are noted. Tissues were stained with Hematoxalin and Eosin (500X).

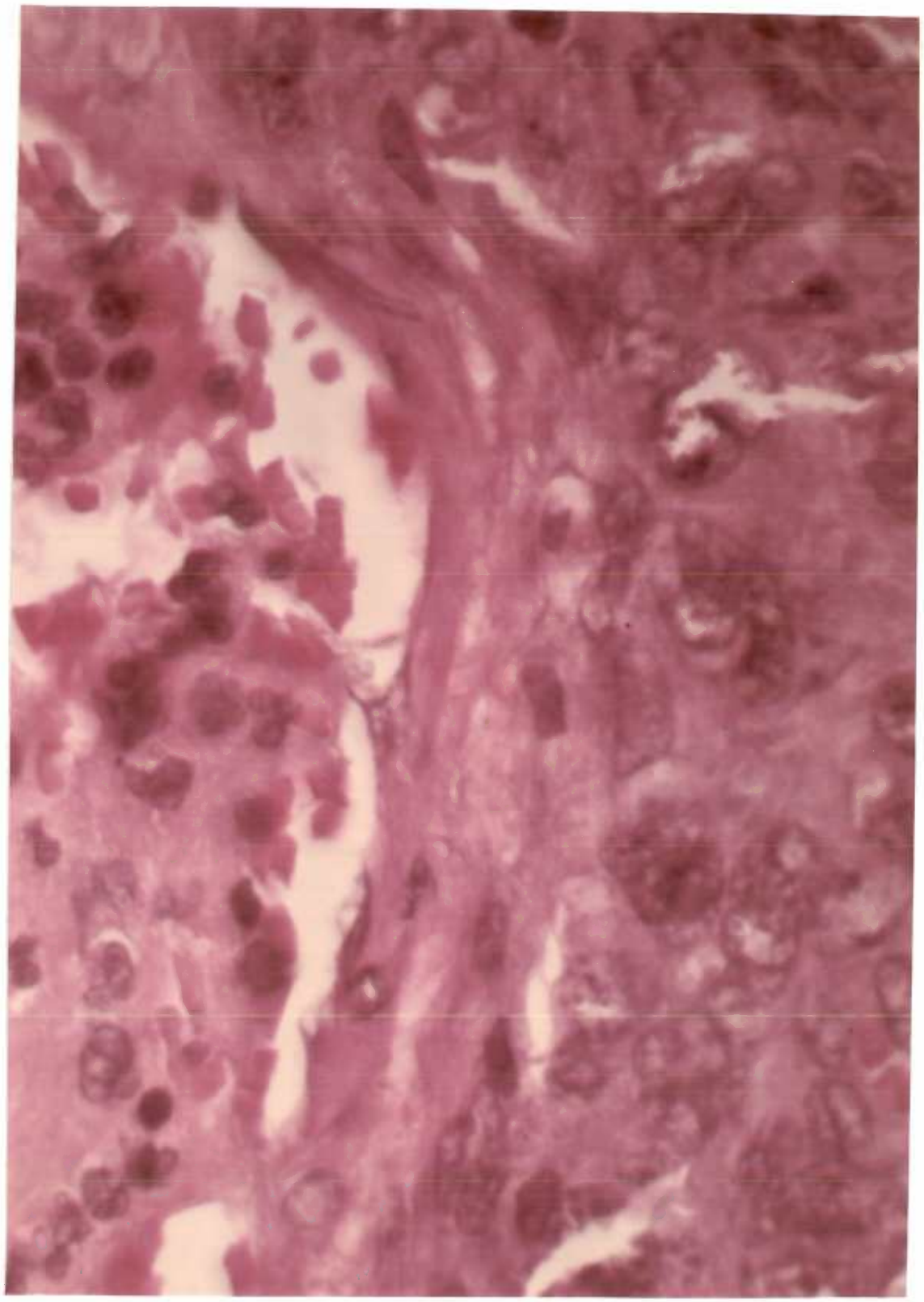


Figure 7

Histological section of MCA-1 tumor tissue. Large, densely granular tumor cells are noted on either side of the central nerve bundle in the center of the field. Tissues were stained with Hematoxalin and Eosin (500X).

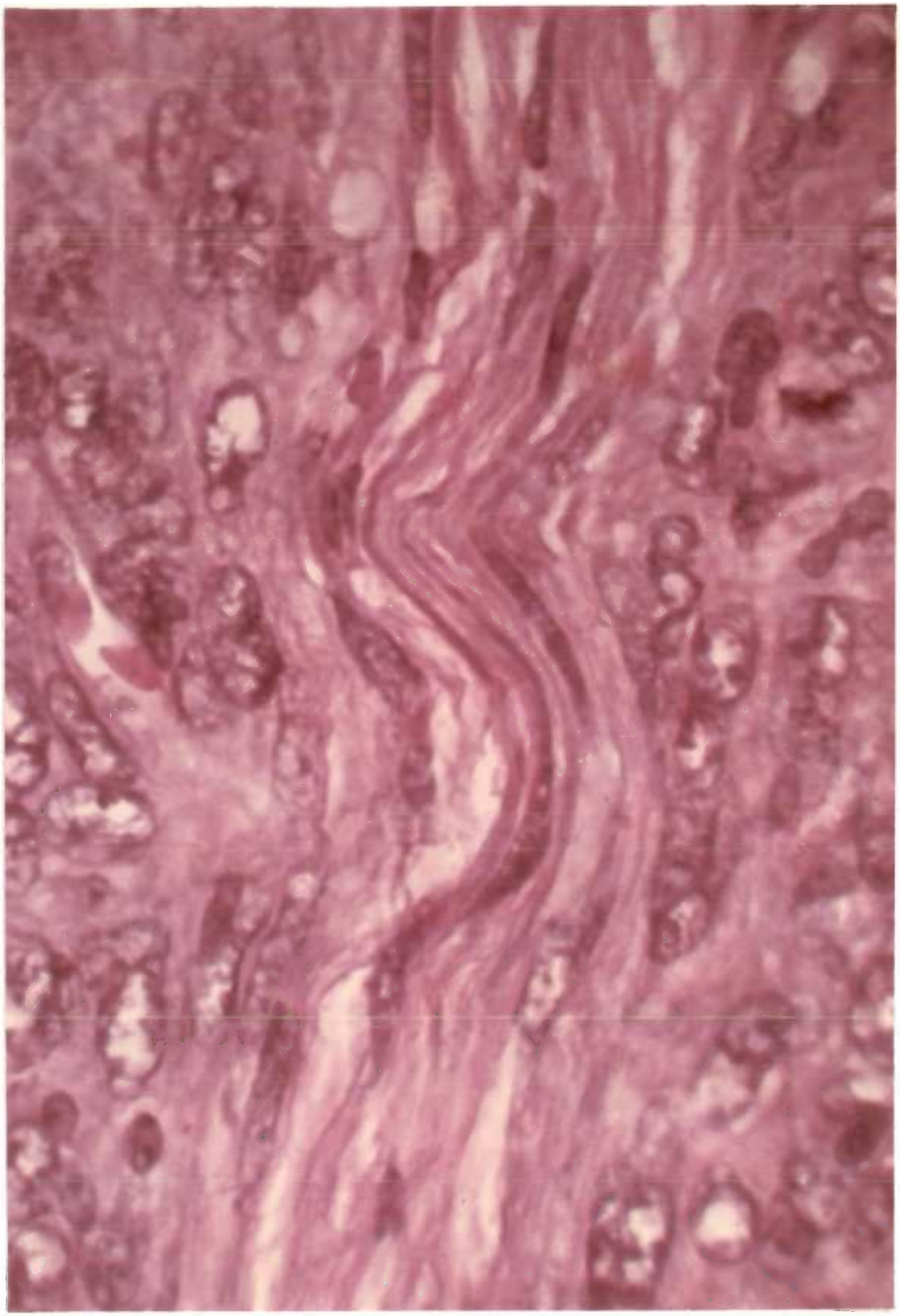


Figure 8 .

Skin test response in a MCA-1 immune guinea pig to TSA. Erythema and induration were noted 24 hours after an ID injection of TSA.

Figure 9

Dose-response relationship of guinea pig whole blood cultures stimulated with varying amounts of phytohemagglutinin (PHA) (0.5 - 30 µg/tube) or pokeweed mitogen (PWM) (1/2000 - 1/30 dilution of stock/tube). Cultures were incubated for 6 days at 37 C with 1.0 µCi ^{3}H-Tdr (sp. act. 6.8 Ci/mmole) added 24 hours prior to harvest.

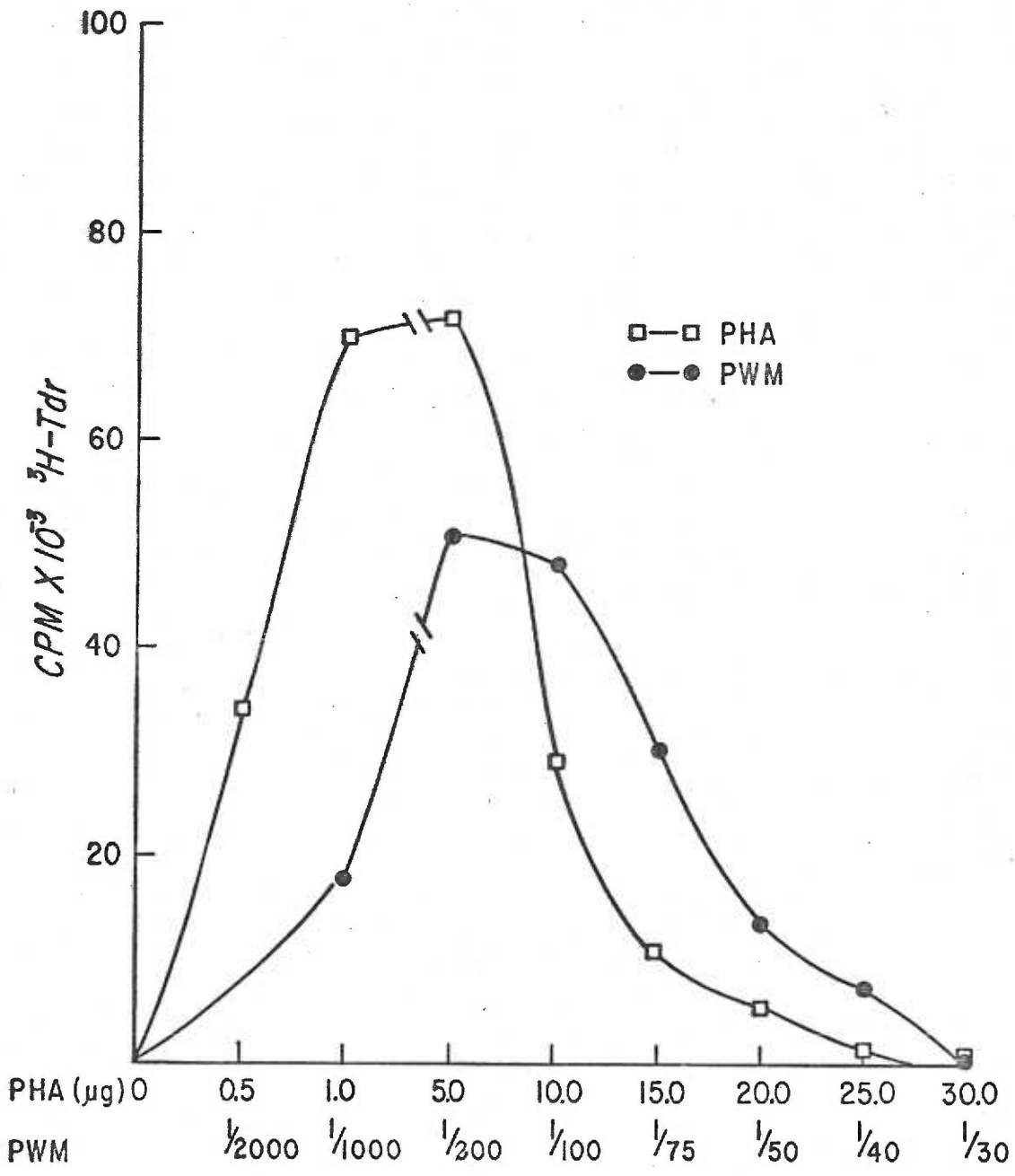

100
80
60
40
20
CPM X 10⁻³ ³H-Tdr
PHA
PWM
PHA (µg) 0 0.5 1.0 5.0 10.0 15.0 20.0 25.0 30.0
PWM 1/2000 1/1000 1/200 1/100 1/75 1/50 1/40 1/30

Figure 10

Relationship between incubation period and response of guinea pig whole blood cultures stimulated with phytohemagglutinin (PHA) or pokeweed mitogen (PWM). Cultures were incubated with 10 µg PHA or 0.1 ml of 1/10 stock PWM at 37 C. Tritiated thymidine (1.0 µCi, specific activity 6.8 Ci/mmole) was added to all cultures 24 hours prior to harvest.

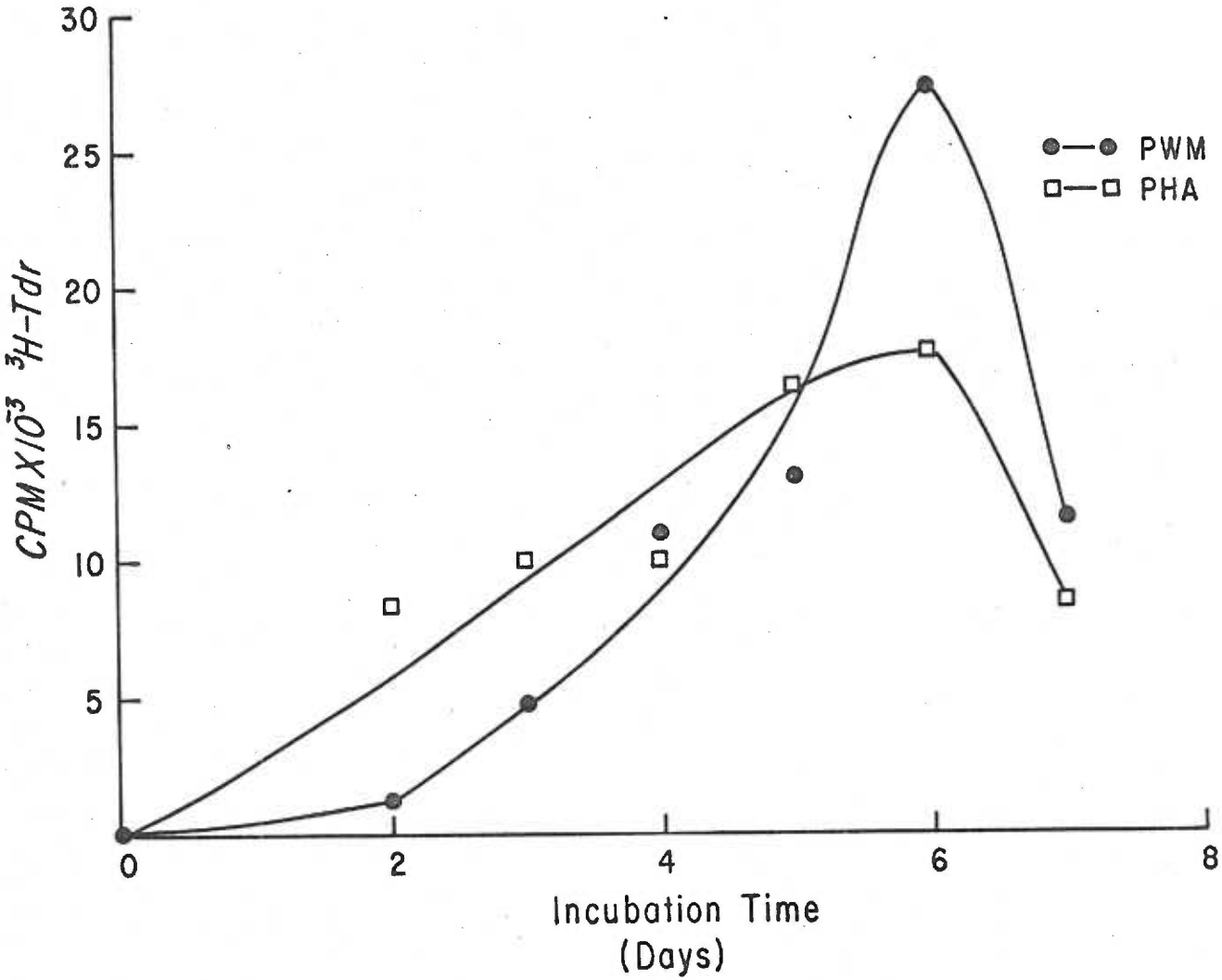
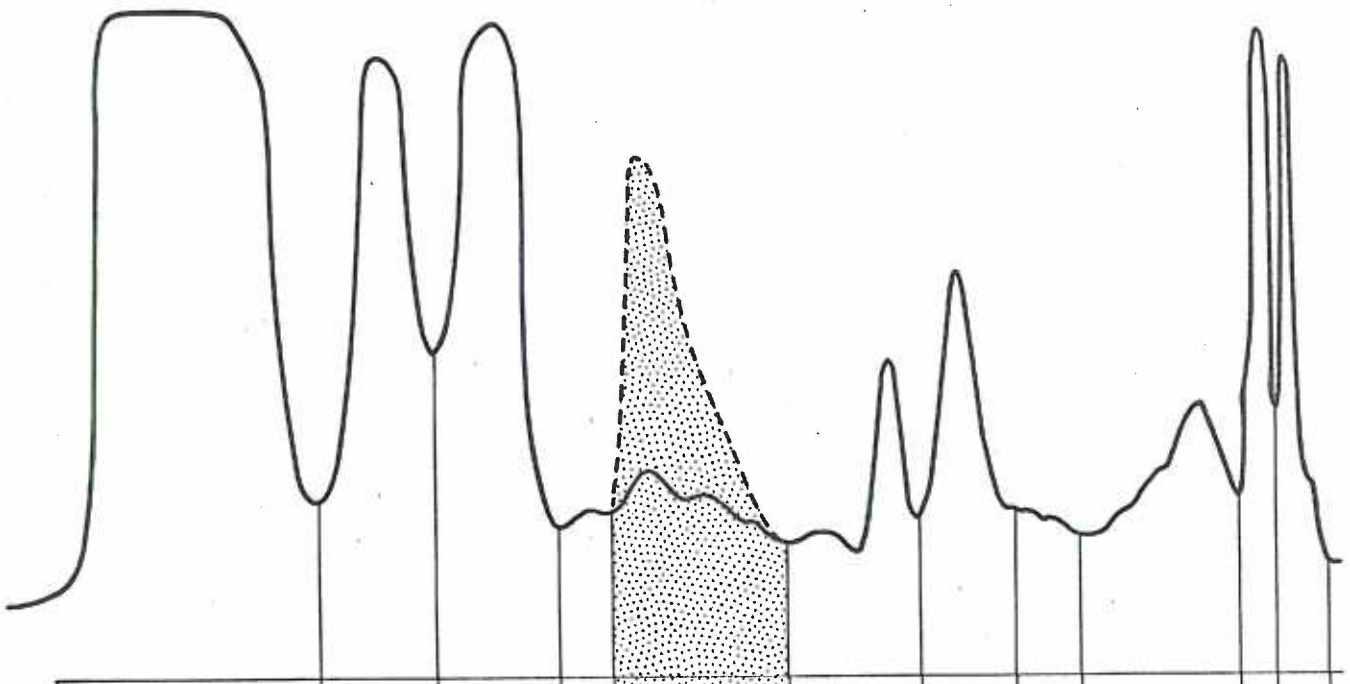

30
25
20
15
10
5
0
2
4
6
8
CPM X 10^3 ³H-Tdr
Incubation Time
(Days)
PWM
PHA

Figure 11

A densitometer scan of a stained polyacrylamide gel after electrophoresis of serum from a control animal. The broken line (---) represents the change in the scan observed from the serum of a tumor-bearing animal. The gel scans of serum from immune animals were indistinguishable from controls. The scans are divided into ten fractions. The percent globulin of each fraction was calculated from the total globulin present by integration. A significant increase ($P < 0.01$) in Fraction 4 was only observed in tumor-bearing animals (students t test).



% Globulin	FRACTION	1	2	3	4	5	6	7	8	9	10
	NORMAL	16.3	24.0	3.5	14.4	7.0	8.8	5.8	11.0	4.9	3.8
	TUMOR-BEARING	14.7	23.5	4.6	18.9	6.0	6.4	5.4	10.8	5.5	3.7
	IMMUNE	14.7	23.0	5.2	13.6	7.2	9.5	7.2	10.6	4.9	3.7

Figure 12

Stained polyacrylamide gels after electrophoresis of serum from normal (N), tumor-bearing (TB) and immune (I) guinea pigs. The arrows designate globulin region 4 which is significantly elevated in the tumor-bearing group when compared to normal and immune groups.

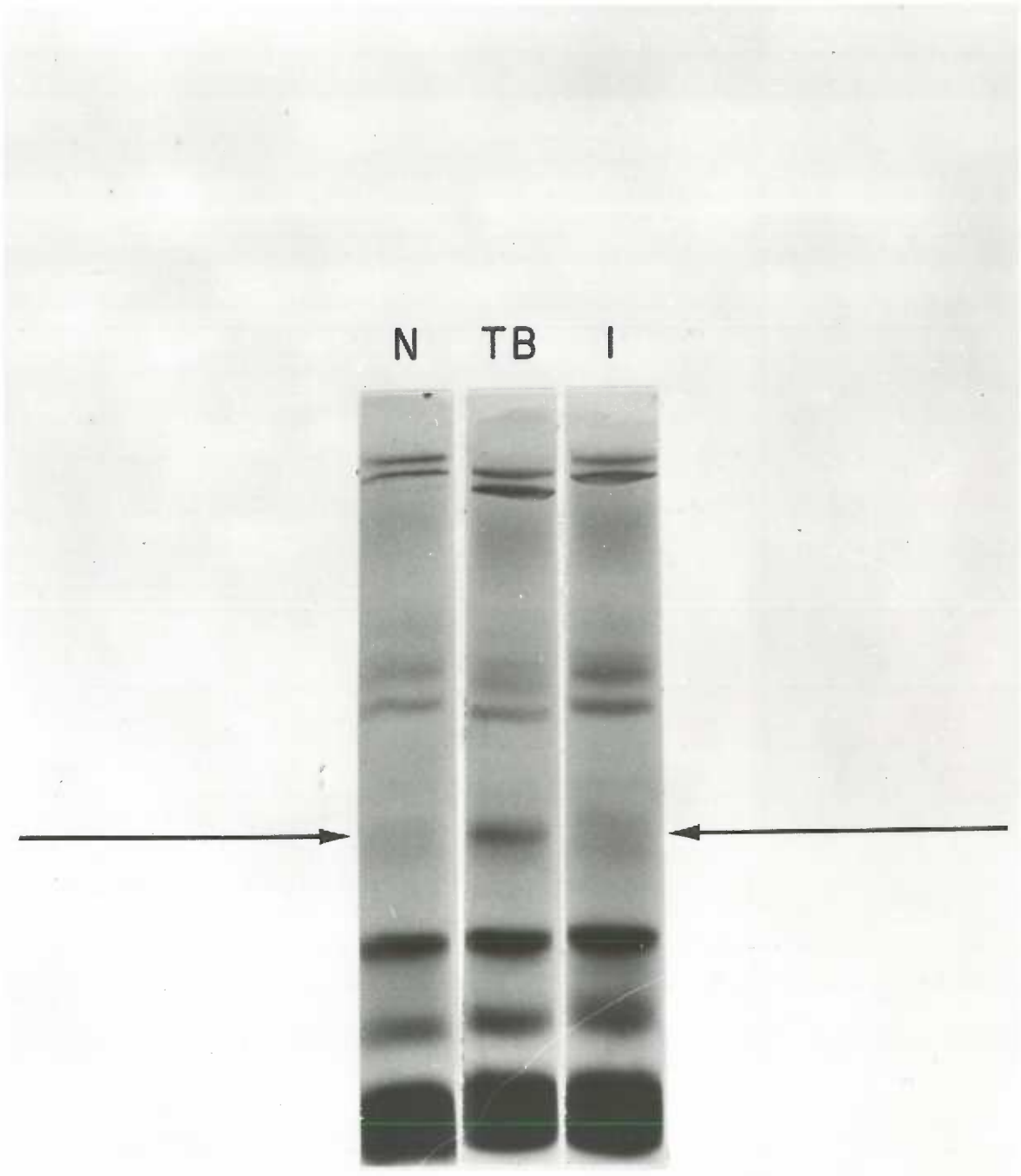

N
TB
I

Figure 13

Stained polyacrylamide gels after electrophoresis of serum from sequential bleedings (1-6) of an animal following tumor inoculation. The arrows designate globulin region 4 which begins to increase 8 days after tumor challenge.


1 2 3 4 5 6
DAY OF
BLEEDING
0 4 8 12 16 20

Figure 14

Relationship between tumor size and relative level of fraction 4 (% of total globulin) in tumor-challenged and control Strain 13 guinea pigs during tumor growth. The levels of fraction 4 of tumor-challenged (--o--) and control animals (—— ● ——) were compared in relationship to tumor size (▥). The fraction in brackets [ ] represents the number of tumor-bearing animals divided by the number of living tumor-challenged animals. Significant differences in F4 levels were observed on days 20, 24, 28 and 32.

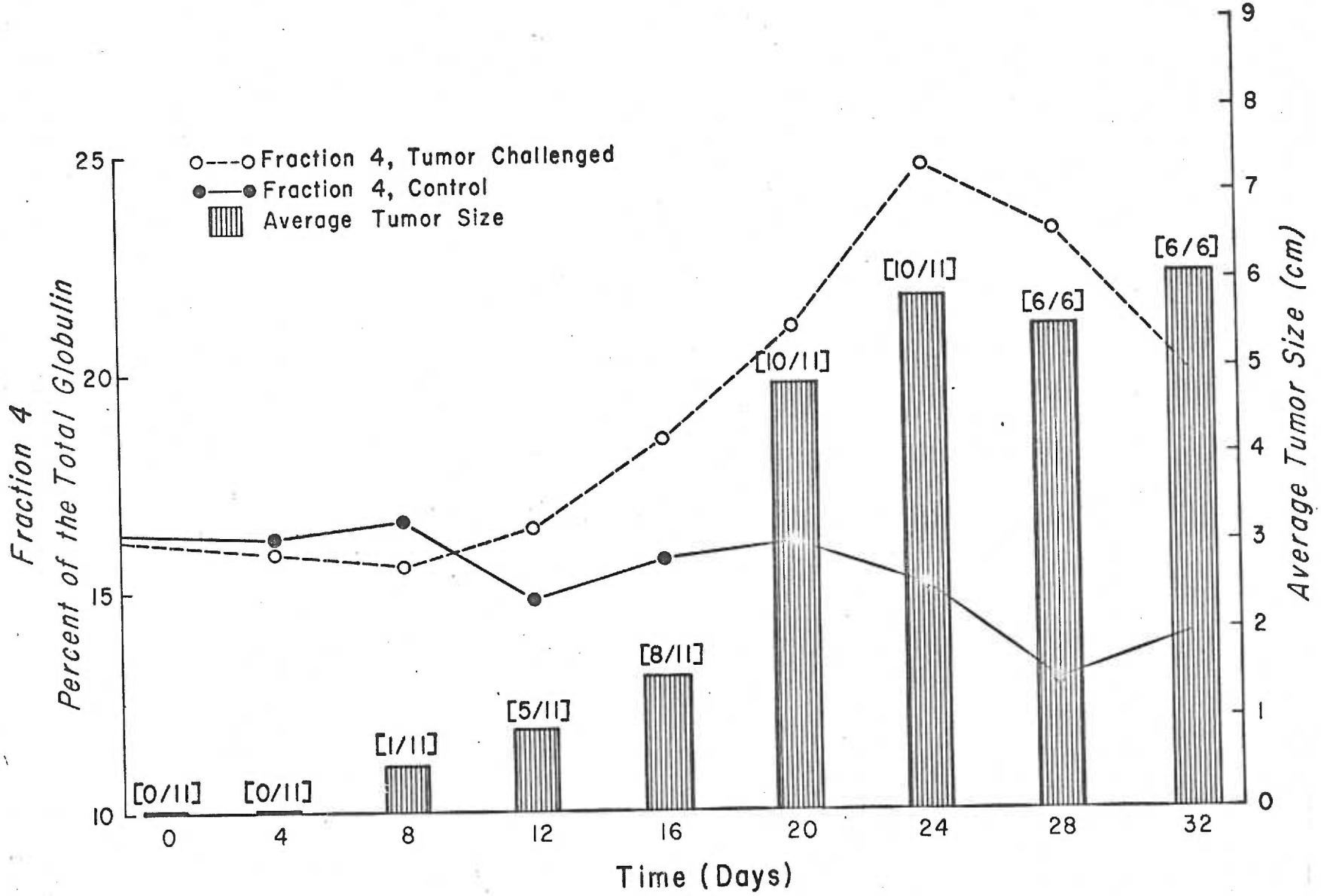

Fraction 4, Tumor Challenged
Fraction 4, Control
Average Tumor Size
Fraction 4
Percent of the Total Globulin
Average Tumor Size (cm)
Time (Days)
[0/11]
[0/11]
[1/11]
[5/11]
[8/11]
[10/11]
[10/11]
[6/6]
[6/6]
25
20
15
10
9
8
7
6
5
4
3
2
1
0
0
4
8
12
16
20
24
28
32

Figure 15

The relationship between tumor size and levels of F4 (%) in 11 animals challenged with MCA-1. The points represent values determined between day 12 after tumor challenge and death of the animals. A correlation coefficient of + 0.9178 ($P < 0.001$) was calculated.

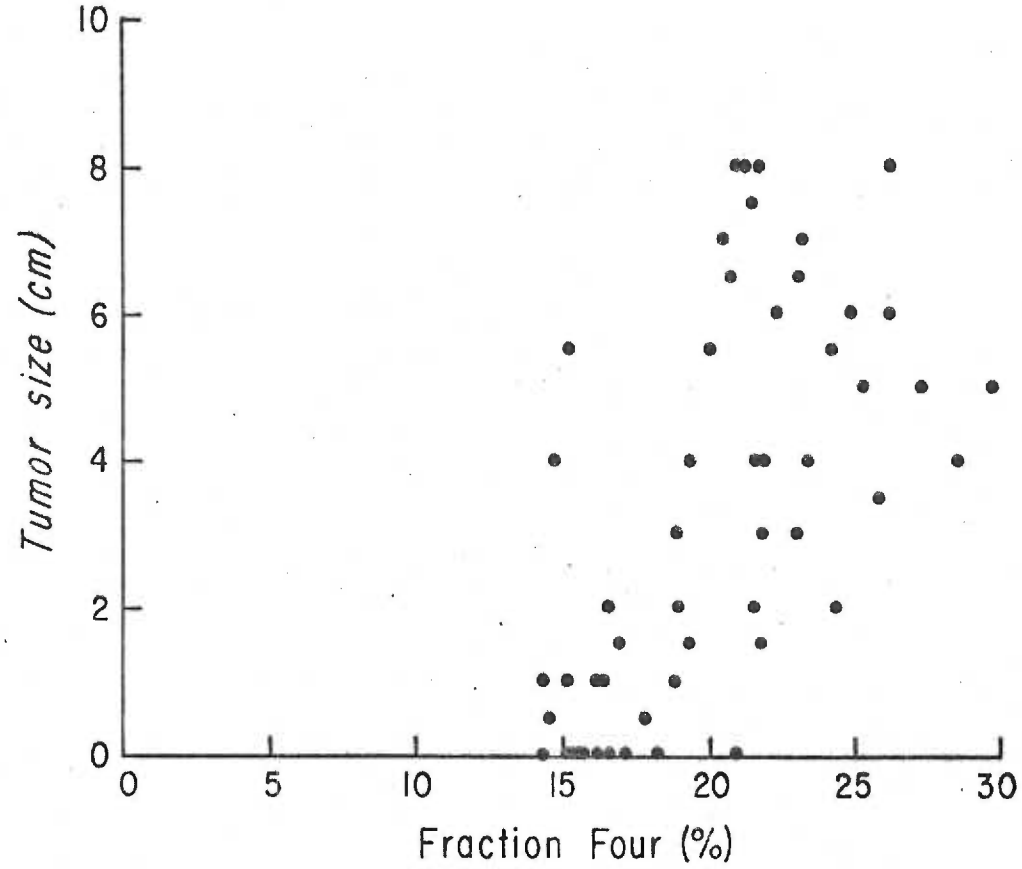
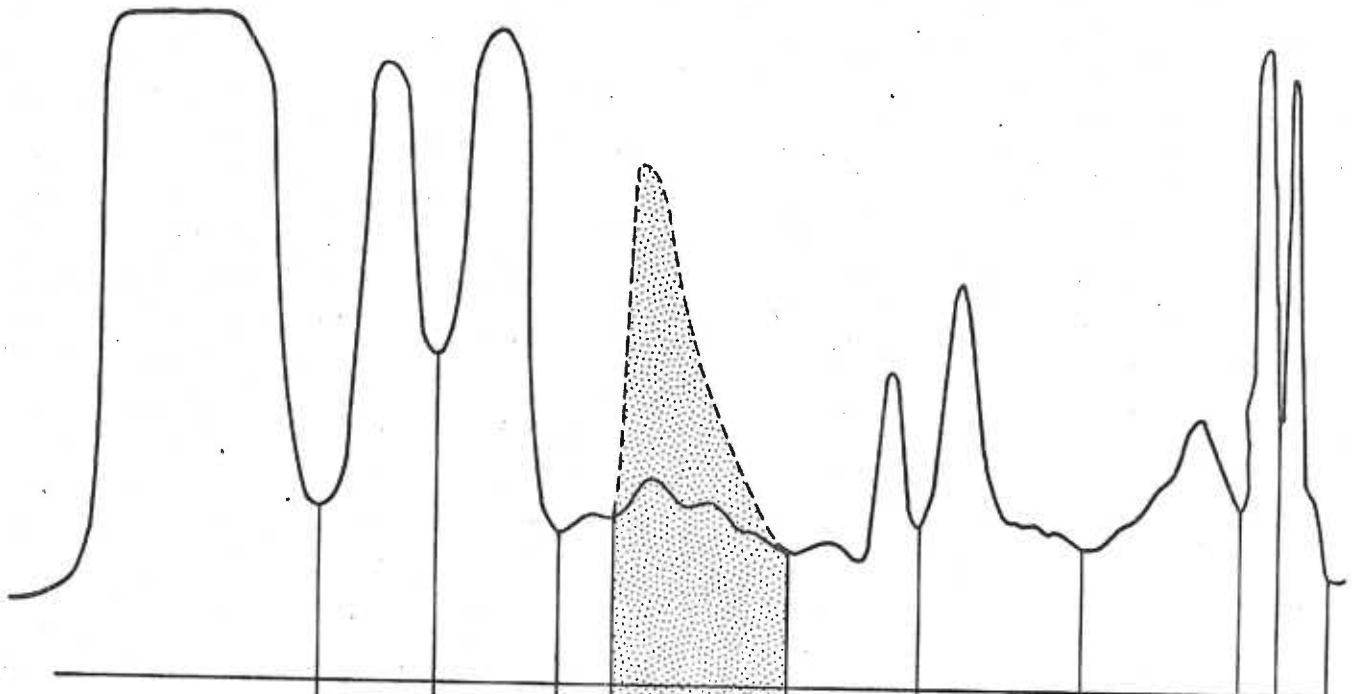

Tumor size (cm)
10
8
6
4
2
0
0
5
10
15
20
25
30
Fraction Four (%)

Figure 16

A densitometer scan of a stained polyacrylamide gel after electrophoresis of serum from a single control group animal. The broken line (---) represents the change in the scan observed when serum from an animal undergoing sensitization to DNFB was electrophoresed (third bleeding). The scan is divided into nine fractions. The percent globulin of each fraction was calculated by integration and is shown for this control animal and one sensitized animal (third bleeding). A significant increase ($P < 0.01$, Students t test) in fraction 4 was observed when comparing ten control and ten test group animals.



FRACTION	1	2	3	4	5	6	7	8	9
% GLOBULIN									
CONTROL	21	22	3	10	6	11	13	7	8
DNFB	18	20	4	17	5	13	10	7	6

Figure 17

A comparison of F4 values of 10 control animals (- - ● - -) and 10 animals undergoing sensitization (—o—) to DNFB (top), 15 animals undergoing sensitiztion to allogeneic skin (middle), and 13 animals undergoing sensitization to Mycobacterium tuberculosis (bottom). A significant increase ($P < 0.01$, Students t test) in this region in animals sensitized to DNFB and allogeneic skin is noted on bleedings two and three. The significant change in F4 levels in the tuberculin group, noted only when the highest F4 values attained were compared to pre-bleed values (Table 3), was not evident at any bleeding during sensitization.

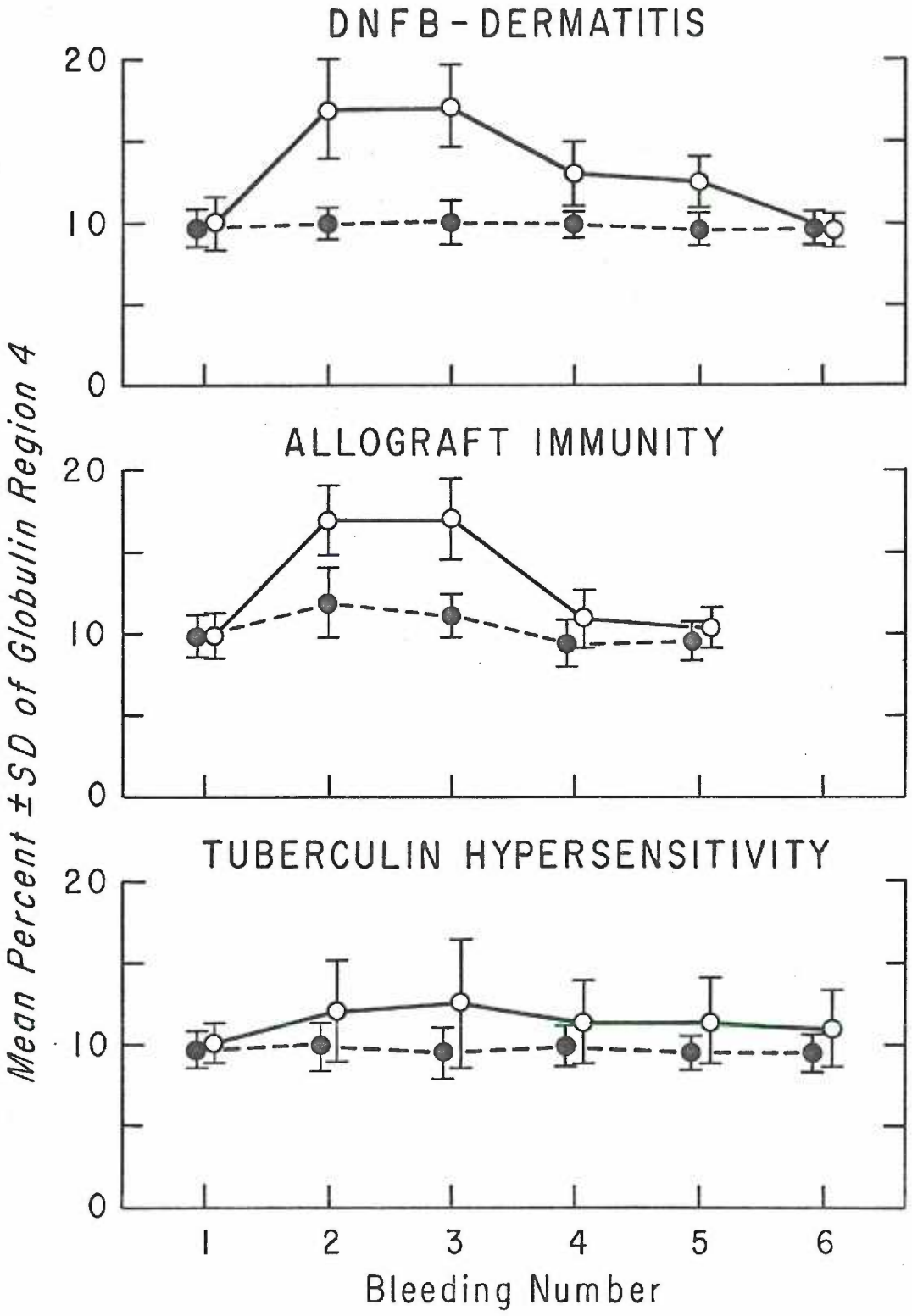

DNFB - DERMATITIS
ALLOGRAFT IMMUNITY
TUBERCULIN HYPERSENSITIVITY
20
10
0
Mean Percent ±SD of Globulin Region 4
1
2
3
4
5
6
Bleeding Number

Figure 18

Stained polyacrylamide gels after electrophoresis of serum from sequential bleedings (1-6) of a typical guinea pig during sensitization to DNFB. The arrows designate globulin fraction 4 which increases to a maximum by the third bleeding and then subsides.

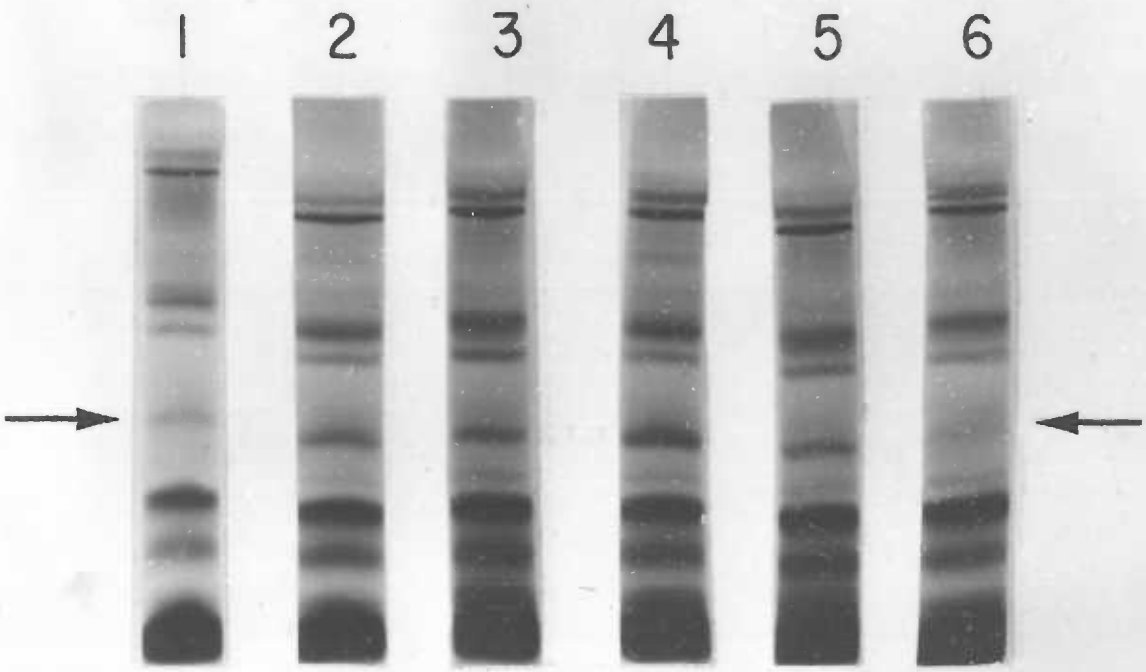

1
2
3
4
5
6

Figure 19

A comparison of serum globulin changes in 15 animals sensitized to allogeneic skin. The shaded areas represent the mean globulin percent ± S.D. of nine electrophoretic fractions of serum from autografted animals (third bleeding). The broken line (---) represents the significant increase ($P < 0.01$, Students t test) in F4 in 15 allografted animals compared to 10 autografted animals at this time.

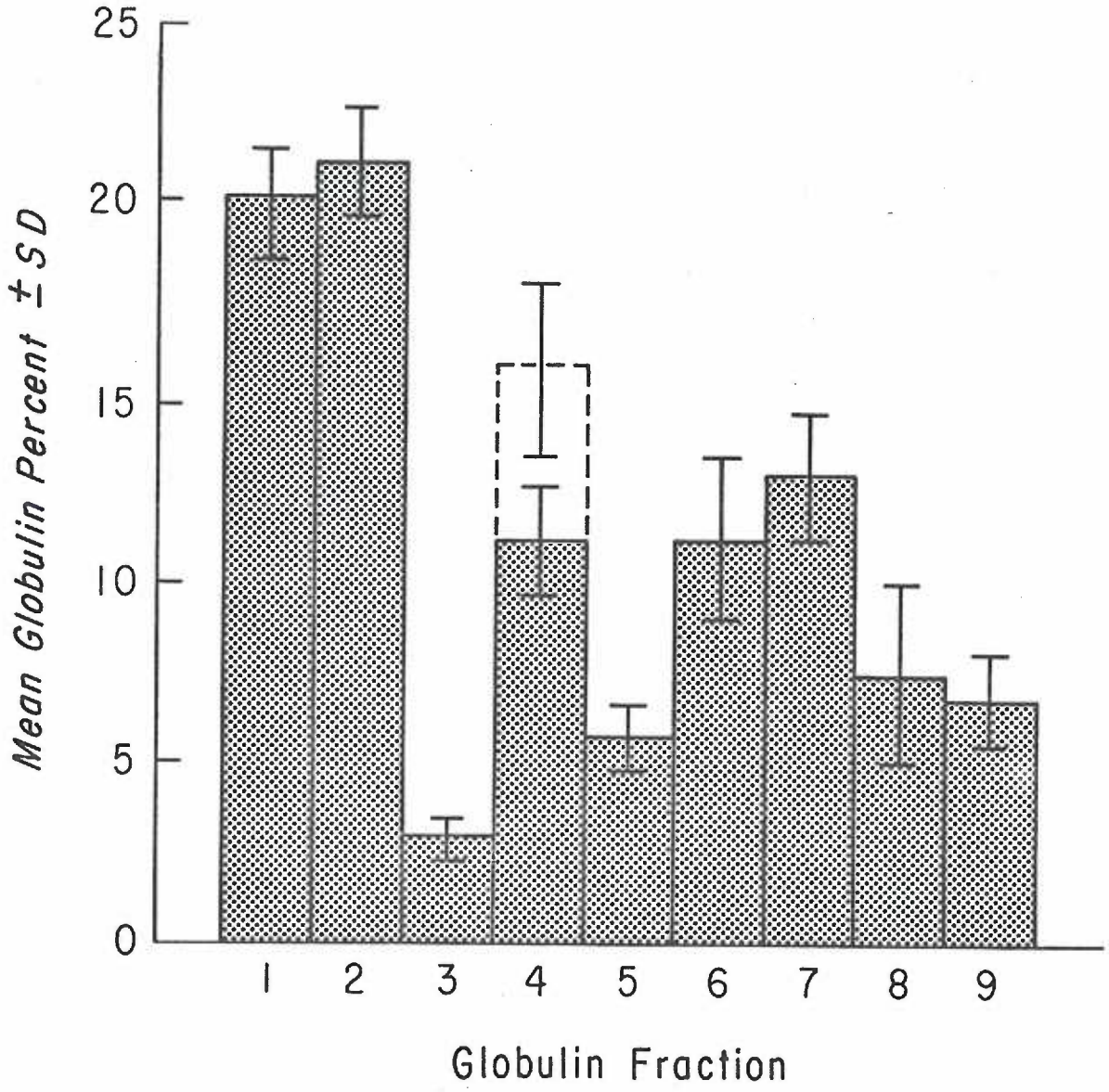

25
20
15
10
5
0
Mean Globulin Percent ± SD
1
2
3
4
5
6
7
8
9
Globulin Fraction

Figure 20

A comparison of PHA or PWM induced lymphocyte responsiveness during tumor growth in Strain 13 guinea pigs. The total number of animals (■) from Experiments I and II are shown. The number of animals with suppressed PHA (□) or PWM (▤) responses are compared during tumor growth. An animal was considered suppressed when its PHA or PWM response was less than 50% of the lowest control value on any one test day.

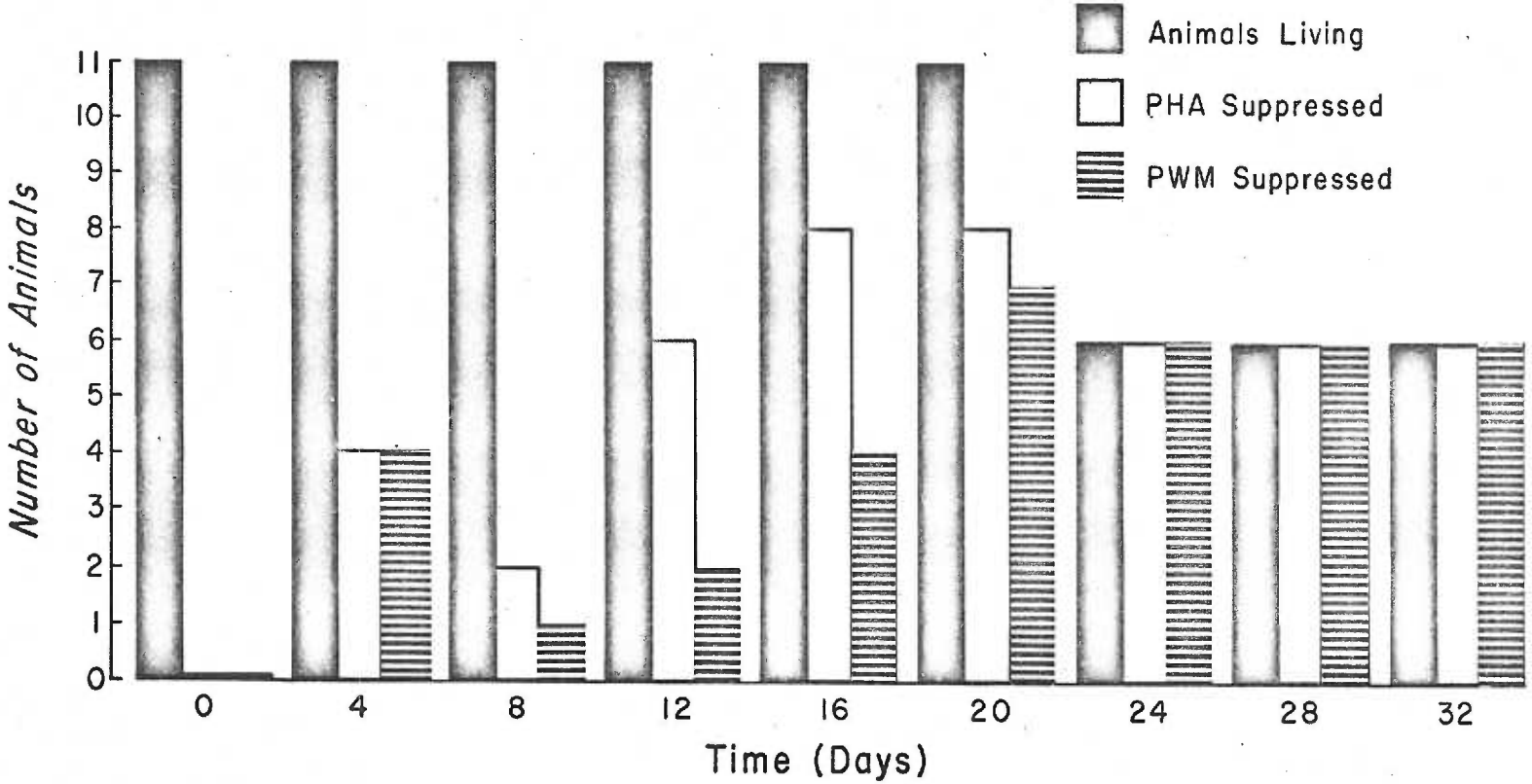

Animals Living
PHA Suppressed
PWM Suppressed
Number of Animals
0
1
2
3
4
5
6
7
8
9
10
11
0
4
8
12
16
20
24
28
32
Time (Days)

Figure 21

Lymphocyte responses to PHA were measured by ^{3}H-thymidine uptake in whole blood cultures in control (18 animals in Experiment I, 16 animals in Experiment II) (open symbols) and DNFB sensitized (18 animals in Experiment I, 16 animals in Experiment II) (closed symbols) animals. The PHA response was significantly suppressed ($P < 0.01$, Students t test) on the fifth day of DNFB sensitization (↓) in separate experiments (-O- , Expt. I, -Δ-, Exp. II). A recovery of the PHA response was noted by day 12.

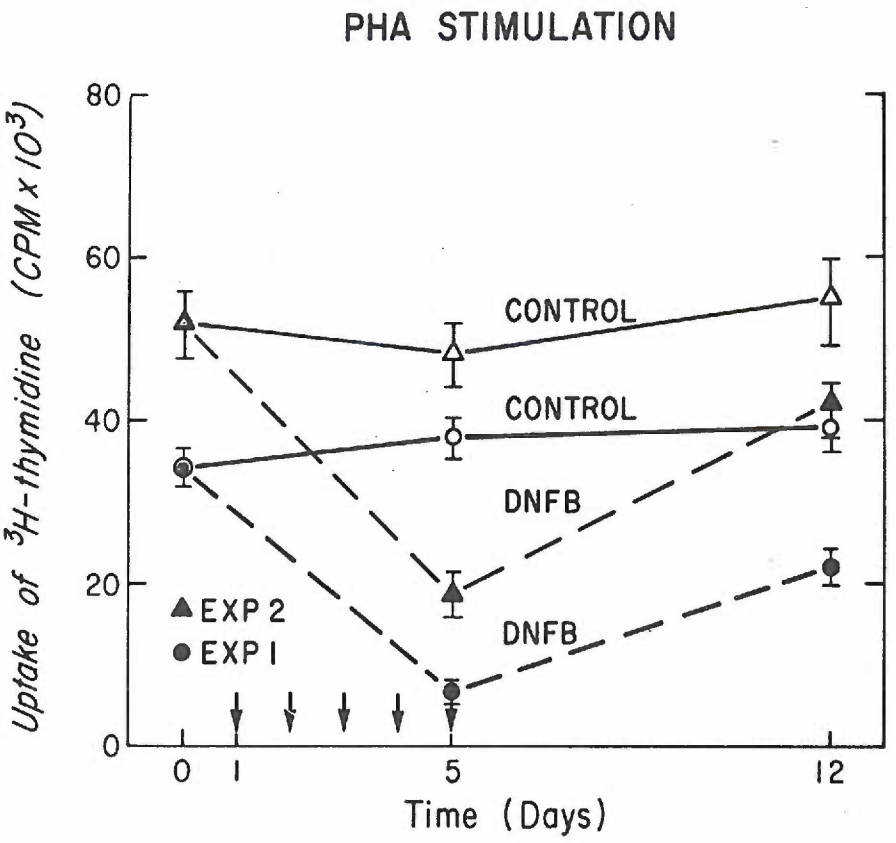

PHA STIMULATION
Uptake of ^3H-thymidine (CPM x 10^3)
80
60
40
20
0
CONTROL
CONTROL
DNFB
DNFB
EXP 2
EXP I
0
1
5
12
Time (Days)

Figure 22

Lymphocyte responses to PWM were measured by ^{3}H-thymidine uptake in whole blood cultures in control (18 animals in Experiment I, 16 animals in Experiment II) (open symbols) and DNFB sensitized (18 animals in Experiment I, 16 animals in Experiment II) (closed symbols) animals. The PWM response was significantly suppressed ($P < 0.01$, Students t test) on the fifth day of DNFB sensitization (↓) in separate experiments (-O-, Exp. I, -Δ-, Exp. II). A recovery of the PWM response was noted by day 12.

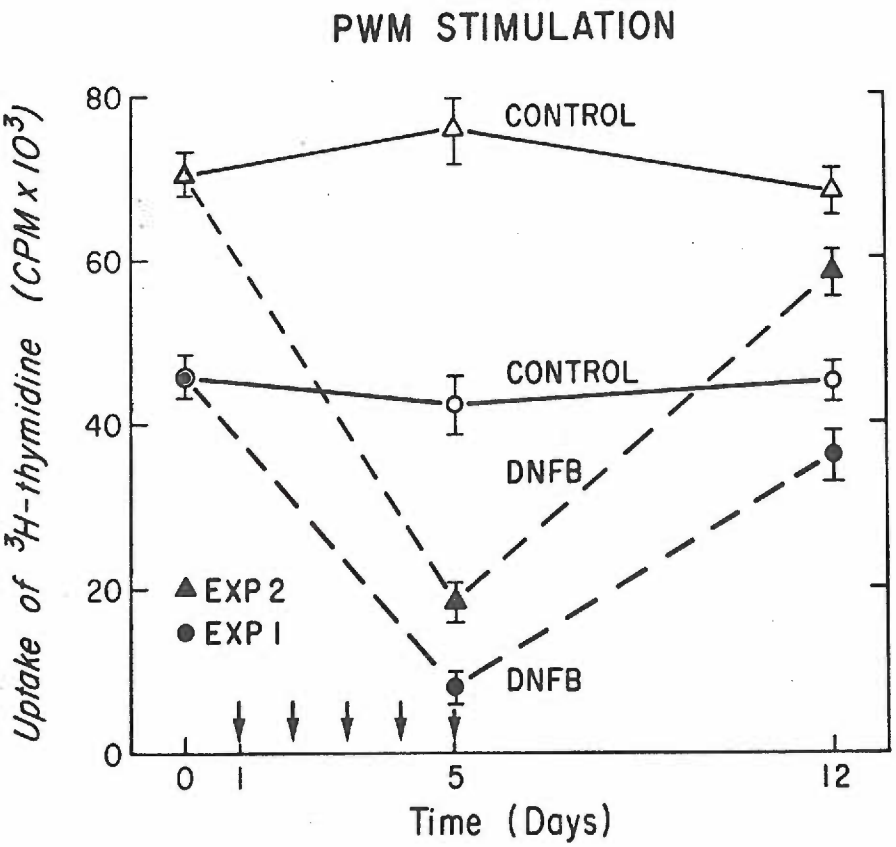

PWM STIMULATION
Uptake of ^{3}H-thymidine (CPM x 10^{3})
80
60
40
20
0
CONTROL
CONTROL
DNFB
DNFB
▲ EXP 2
● EXP 1
0
1
5
12
Time (Days)

Figure 23

Lymphocyte responses to Con A were measured by ^{3}H-thymidine uptake in whole blood cultures in control (16 animals) (-O-) and DNFB sensitized (16 animals) (-●-) animals. The Con A response was significantly suppressed ($P < 0.01$, Students t test) on the fifth day of DNFB sensitization (↓) in a single experiment. A recovery of the Con A response was noted by day 12.

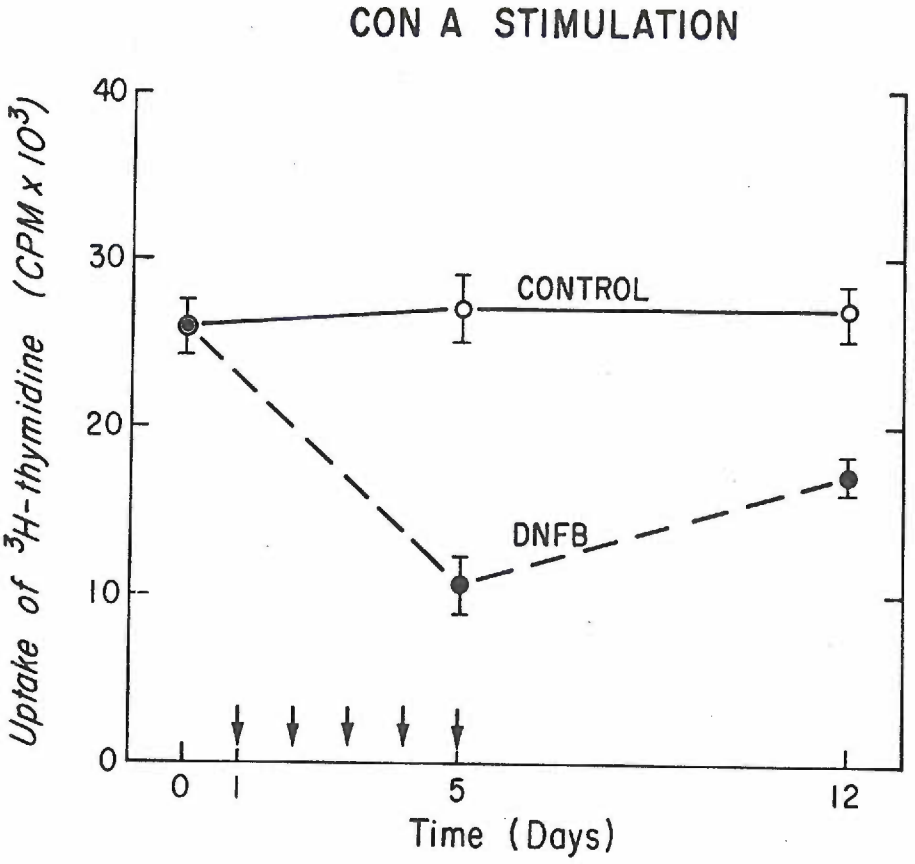
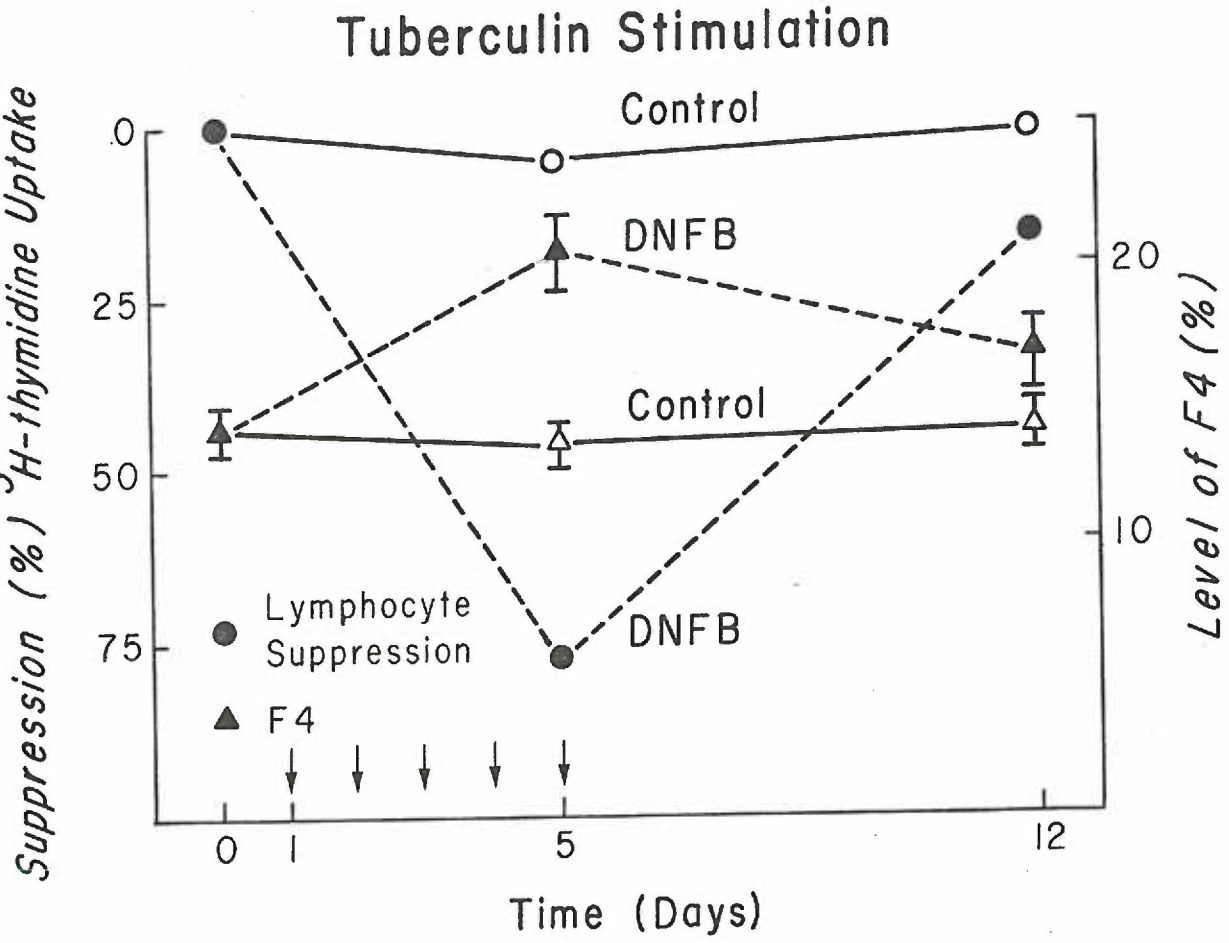

CON A STIMULATION
Uptake of ^{3}H-thymidine (CPM x 10^{3})
40
30
20
10
0
CONTROL
DNFB
0
1
5
12
Time (Days)

Figure 24

Tuberculin (PPD) induced lymphocyte stimulation was measured in tuberculin sensitive animals before (Day 0), during (Day 5), and after (Day 12) DNFB sensitization (↓). Significant suppression was observed on Day 5 in DNFB treated (—●—) but not control (—○—) animals. Rise of fraction 4 (-▲-), elevated during sensitization to DNFB, was not increased in control animals (-△-).


Tuberculin Stimulation
Control
DNFB
Control
DNFB
Lymphocyte Suppression
F4
0
25
50
75
20
10
0
1
5
12
Time (Days)
Suppression (%) ³H-thymidine Uptake
Level of F4 (%)