IMMUNOLOGY OF SPONTANEOUS TUMORS

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1. Introduction

The central question underlying the recent upsurge of interest in the field of "tumor immunology" is whether neoplastic cells possess antigens unique to the neoplastic condition, that is, antigens not shared by the normal cells of the same organism. The reasons for the considerable interest expressed by biologists and clinicians in this question are obvious. If neoplastic cells do indeed possess unique antigenic characteristics, a potentially very promising area of preventive and therapeutic measures in human malignancy would be indicated. In addition, the recognition of qualitatively new macromolecular structures or organizations of the cellular components of tumor cells could provide new tools for studies of the basic nature of neoplastic transformations and progression.

Attempts to detect cancer specific antigens were initiated early in the development of experimental tumor biology. The motivating assumptions for these studies appear reasonable even in retrospect: the progress of neoplastic diseases in man and in animals does not suggest that the host of a population of neoplastic cells plays an entirely passive role in such, essentially parasitic, relationships. Quite to the contrary, the natural history of many neoplastic diseases, with their repeated periods of remission and exacerbation, often related to events which affect the physiology of the host, points to an active role by the host animal. That this role may well be of immunological nature (that is, the formation of specific circulating antibodies and of specifically sensitized cells) also seems reasonable: neoplastic cells appear and behave differently than do corresponding normal ones; differences in morphology and function are often indicative of differences in macromolecular composition or arrangement; and it is well recognized that even very minor changes in the structure or arrangement of molecules can result in new antigenic specificities.

Against this argument there must be counterposed another one, however: effects on animals which are probably not accompanied by significant changes in immunological capability also influence the progress of neoplastic diseases; and the altered appearance and behavior of neoplastic cells *could* accrue from quantitative, rather than from qualitative, changes in the nature of the cell

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components. It is thus apparent that strong experimental evidence is required to permit even the inference that tumor immunology is, in fact, an existing area of investigation.

The experimental evidence until 1953 is, unfortunately, of little value. Many of the studies did indeed show that animals reacted with heightened resistance upon second exposure to implants of neoplastic tissue, suggesting that the initial tumor experience evoked a degree of immunity. It has become clear during the past 15 years, however, that the factors which govern the acceptability of both normal and neoplastic grafts are largely immunological in nature (assuming, of course, that the anatomic and physiologic conditions are appropriate for the acceptance of the graft), and that, barring certain exceptional circumstances, tissue grafts between individuals will be accepted only if the antigenic composition of the tissues of donor and host are essentially identical [1], [2], [3]. (The immunological rejection of foreign tissues is known as the homograft reaction.) This condition requires, in turn, that tissue donors and hosts stand to each other in very close genetic relationship, approximating that of identical twins. Strains of animals of such genetic, and hence antigenic, similarity can be created, by consecutive brother-sister matings over many generations [4], [5], [6]. Such strains are spoken of as isogenic; the criterion of isogenicity is the permanent retention of second set grafts of skin and other normal tissues in a condition identical with that of an autograft [7]. Rejection of a graft of neoplastic tissue within such a strain of animals provides presumptive evidence that the neoplastic tissue possessed unique antigens. In contrast, if isogenicity of the test animals is not clearly established, rejection of a tumor graft may merely indicate that the donor tissue, quite irrespective of its neoplastic nature, was genetically, and hence antigenically, foreign to the host. Almost all of the experimental evidence until 1953 which purported to show the existence of specifically acquired tumor resistance, and hence the existence of tumor specific antigens, founders on the failure of the investigators to use isogenic strains of animals, or to provide evidence for the *bona fide* isogenicity of supposedly inbred strains.

In addition to transplantation studies, another model was used in the early attempts to detect tumor specific antigens. This consisted of immunizing animals with neoplastic tissues derived from another species or strain, absorbing the resulting sera with normal tissues supposedly of the same genetic and antigenic composition as the neoplastic one, and then examining the sera for residual antitumor reactivity. The results of such experiments were also claimed to demonstrate the existence of tumor specific antigens, but they, too, remain equivocal. For one, there are serious technical difficulties in bringing about complete removal of antibody by absorption, and few of the earlier investigators of the phenomenon of tumor immunology appear to have taken seriously Woglom's warning that "Far too large a proportion of cancer immunity experiments are merely ridiculous, for few investigators seem to realize that cancer research is a discipline requiring some apprenticeship, and that not everyone with an inoculating needle and a dozen white mice can plunge in and emerge with a

discovery" [8]. The difficulties are compounded when the immunizing tissue is neoplastic and the absorbing tissue normal. It is known that the complement of *normal* antigens which characterize strains and specific tissues may be grossly different in neoplastic and in normal cells [9], [10]. The investigator may thus fail to achieve complete removal from the immune serum of large quantities of antibody directed at certain *normal* tissue components by absorbing with normal tissues, and may draw the erroneous conclusion that he has discovered a tumor specific antibody. Moreover, it has been shown that neoplastic cells sometimes contain or elaborate antigens which are characteristic of the fetal stage of that organ [11]. It is not known whether an adult animal can respond immunologically against its own fetal antigens, and the clinical implication of their presence in tumor cells is therefore uncertain; it is clear, however, that such fetal antigens represent tumor specific antigens only in a very special sense. A second major difficulty with the earlier serological experiments again arises from the lack of proven isogenicity of the test animals; thus, the immunizing neoplastic tissue could have contained normal tissue antigens not possessed by the absorbing normal tissue.

The criticisms here leveled at the earlier work in tumor immunology remain pertinent to many of the more recent experiments. However, Foley's demonstration in 1953 [12], that mice of a strain clearly shown to be isogenic were able to develop significant degrees of heightened resistance against implants of sarcomas induced by a carcinogenic hydrocarbon as result of a previous, experimentally terminated experience with the tumor, brought tumor immunology into the area of respectable scientific inquiry. Since then, a considerable body of evidence has accumulated in support for the operation of immunological defenses against neoplastic cells. This evidence comes largely from transplantation studies with tumors induced experimentally with chemical carcinogens [13] to [20], cellophane films, and polyvinylchloride platelets [18], [21]; initiated in the laboratory by oncogenic viruses [22] to [32]; or developing after exposure to UV rays [33]. In addition, persuasive evidence for the presence of tumor specific or tumor associated antigens in a number of cancers induced in the laboratory by oncogenic viruses has recently come from experiments with sera and lymphoid cells of animals immunized with the tumors [34] to [41]. It has also been suggested that there may be an immunological basis for the occurrence of noninfective Rous sarcomas in chickens [42] and for the spontaneous regression of Shope papillomas in rabbits [43]. Several recent reviews of the tumor immunology literature are available [44], [45], [46].

Henceforth in this communication, the term tumor specific will be applied to an antigen only when such antigen is found exclusively on or in fully neoplastic cells. The broader term tumor associated will be used to designate special antigens held by *both* neoplastic and preneoplastic cells, including those antigens of neoplastic cells which are possessed or induced by an oncogenic virus and which are expressed in infected preneoplastic and normal cells as well.

It may now be asked why cancer cells, if they are indeed marked by a specific

new antigenicity, succeed in establishing themselves in the tissues of their host and there are able to grow progressively. Why doesn't the host reject them immunologically, as foreign tissue? Several answers may be suggested. A rapidly growing population of cells may succeed in establishing itself progressively *despite* immunological attack and attrition by the host. Large quantities of tumor specific antigens may be produced rapidly, and induce a state of specific immunological unresponsiveness. (It is a peculiarity of the immune response that large quantities of antigen can somehow inhibit the specific response [47], [48], [49].) It may also be that neoplastic mutants in fact arise frequently in higher animals, but are suppressed immunologically, and that it is only the individual suffering from a congenital or acquired (as with advanced age) immunological disability who permits the progressive development of the neoplastic cells. It has, indeed, been suggested that a major function of the immune response may be the destruction *in situ* of neoplastic mutants [50], and there exists a body of circumstantial evidence pointing to an inverse relationship between immunological reactivity and tumor susceptibility [51].

There is thus no irreconcilable paradox between the fact of tumor antigenicity and the fact that neoplastic cells, once they are detectable, commonly continue to grow until death of the host. What does remain uncertain from the evidence cited so far is whether the antigenicity now established for almost every category of *experimental* tumors permits extrapolation to the situation of tumors which arise *spontaneously* or "naturally," that is to say, without known manipulation on the part of the observer. Very little work has been done so far on the immunology of spontaneous tumors, and still less with spontaneous tumors of recent origin. There are good reasons for this—it is much easier to work with established, well characterized lines of cells, whose neoplastic state is predictably inducible by certain means—but the fact remains that persuasive arguments can be advanced that the antigenicity, or immunogenicity, of experimentally induced tumors represents an artifact, and cannot be taken as a model for neoplasia in nature. (Most of the evidence for the existence of tumor specific antigens comes from transplantation studies. In many recent studies of this nature, the conditions have been such as to satisfy the criteria of an immunological reaction. However, the antigens responsible for the transplantation resistance phenomena have not been isolated and characterized, and it would, accordingly, be more appropriate to speak of "immunogenicity" than of "antigenicity." The terms "immunogenicity" and "immunogen" and "antigenicity" and "antigen" are nonetheless employed interchangeably in the transplantation literature, and in order to avoid the creation of new semantic strictures they will be used interchangeably here when reference is made to experiments in which heightened tumor reactivity can be reasonably assumed to rest on immunological mechanisms.) The arguments are as follows: certain carcinogenic agents and conditions (such as UV and X-irradiation) act also as mutagenic stimuli. The antigenicity of clones of neoplastic cells arising as consequence of exposure to

such agents or conditions may result from the mutagenic effects, and some of these may be causally unrelated to the carcinogenesis. It is also known that many carcinogenic chemicals and conditions act to depress general immunological capability [52]. This property may be independent of their carcinogenic action, and the antigenicity of the resultant neoplastic cells may arise from the fact that the immunologically depressed host permits the establishment of mutants within the population of induced neoplastic cells, which are marked by antigenic novelty. (It must be noted that another correlation between the carcinogenic and immunity depressing qualities of carcinogenic stimuli has been advanced: it could be that the carcinogenic properties of such substances and conditions actually *accrue* from the depression of immunological responsiveness, creating thereby an environment in which naturally occurring neoplastic cell variants, by nature antigenically distinct, are allowed to develop [52].)

Lastly, many tumor associated antigens are antigens possessed by the oncogenic virus which has initiated the neoplasia, or which are induced by action of the virus on the host cells. When the oncogenic virus is one maintained in the laboratory for long periods of time, and selected for specific oncogenic properties, the antigenicity which it controls may be as artifactual as the nature of agent, that is, the antigenicity of tumors induced by laboratory strains of viruses cannot be taken as presumptive evidence for the antigenicity of tumors induced in nature by wild oncogenic viruses.

It was with the aim of attacking the question of tumor specific or tumor associated antigenicity in spontaneously occurring tumors that we initiated, five years ago, an intensive study of the immunology of spontaneous mammary carcinomas of mice.

Evidence that spontaneous tumors possess antigens specific to, or associated with, the neoplastic condition was very sparse when our study was begun. The presence of such antigens had been indicated in certain spontaneous mouse leukemias by Gorer, Tuffrey, and Batchelor [53]. Hirsch and coworkers had reported a "small but significant" increase in the survival time (an unsatisfactory criterion!) of mice given an implant of a newly arisen mammary adenocarcinoma immediately after removal of a first, immunizing implant [54], and Koldovsky had claimed success in eliciting acquired immunity against spontaneous mammary carcinomas in mice of "controlled antigenic homogenicity" [55], [56], [57].

2. Spontaneous mammary carcinomas of mice

Spontaneous mammary tumors of mice were chosen as the model to be investigated for several reasons. They are readily available in a number of inbred strains of mice, which differ from one another in many other respects. There is considerable knowledge of the biology of these tumors and of the host species, and, very importantly, for reasons which will become apparent later, a pre-

neoplastic stage in the progression of normal mammary parenchyma to mammary carcinoma has been discovered and characterized. The major reason, however, for choosing this tumor system for intensive immunological investigation arose from its similarity, in a number of important aspects, to neoplasia in nature.

Spontaneous mammary carcinomas develop in wild and in laboratory strains of mice without the intentional agency of the investigator, except that certain strains have been inbred for high or low mammary tumor incidence. As in most of the neoplasias for which inciting factors have been identified, viral agents play an important role in the etiology of mouse mammary cancers. In contrast to most of the virus induced tumors studied in the laboratory, however, mouse mammary tumors are not induced by artificial introduction of strains of virus selected by the experimenter, but rather as a result of naturally occurring infection through ovum, sperm, or milk with viruses endemic within certain strains and families of this species. The interaction of virus and host which leads to the development of the preneoplastic condition of the mammary gland, the hyperplastic alveolar nodule, or HAN [58], and subsequently to the neoplastic state, is very complex. Hormonal, and other physiological, factors influence significantly the noduligenic, and possibly also the tumorigenic, potential of the mammary tumor viruses (designated MTV, Mammary Tumor Virus, and NIV, Nodule Inducing Virus), and mammary carcinomas sometimes occur in mice not infected with these agents. The cancers usually develop relatively late in life, at a time when the immunological reactivity of the mouse seems to be no longer maximal [59], [60], [61]. There are considerable variations in the age of onset of detectable mammary carcinomas in individual mice, even within the same inbred strain, and there are also considerable differences in the course of the disease. Spontaneous regressions are rare, but they do occur [62]. Further similarities between the mouse mammary carcinoma and spontaneous cancers in noninbred animals and in man could be cited.

With the several rather tenuous exceptions cited above, mammary carcinomas of mice were generally considered to be nonimmunogenic at the time at which our study was initiated. This climate of opinion was generated by the failures of other investigators to demonstrate their immunogenicity in experiments in which simultaneoulsy tested carcinogen induced tumors *were* found to be highly antigenic. It was also believed that if mammary tumors *could* be shown to be antigenic, and an adequate explanation be provided for the several reported failures to detect their antigenicity, a more solid basis would be created for the view that tumors occurring in nature are distinguished from the normal tissues of their hosts by antigenic characteristics. The results of our study, as well as those carried out independently by Morton [63], [64], [65], have shown that mammary carcinomas of mice do, indeed, possess strong tumor *associated* antigens; evidence is developing currently that these tumors may also have true tumor *specific* antigens; and an explanation for the inability of other workers to reveal this antigenicity has come to light.

3. The use of recently arisen tumors and the isogenicity of the test animals

A very large proportion of studies in tumor biology, including tumor immunology, are still conducted with tumors which have arisen years, and sometimes decades, earlier, and which are maintained by repeated passage in "bank" animals or in tissue culture. The advantages of using such "standardized" populations of neoplastic cells are apparent, but the disadvantages are also considerable, and they justify the opinion that results obtained with such populations of cells really do not permit inference to tumor cells of recent origin. This is especially true for studies in tumor immunology.

It is known that drift of isoantigenic characteristics occurs commonly in inbred mice over long periods of time. The isoantigenic characteristics of a strain at the time of testing may therefore be significantly different from those prevailing when a tumor first arose, years previously, within that strain. It has also been shown that tumor cells may lose isoantigens in the course of animal passage [66], and that most or all of the antigens deleted may be lost during the first several transfers [67]. The resulting changes in cell surface characteristics may affect the acceptability of the cells by isogenic hosts for nonimmunological reasons [46], [68], and also for immunological ones not associated with the presence of tumor associated or tumor specific antigens: the loss of cell surface constituents may result in the greater "exposure" of other, weak transplantation antigens whose nonisogenicity within a supposedly isogenic population may have gone undetected; it is also conceivable that membrane components which are normally not exposed will be treated as "new," that is, as antigens, by the animals' immunological apparatus once they are unmasked. (It does not appear possible to rule out entirely this eventuality even in experiments carried out in the primary autochthonous hosts, that is, the animals in which the tumors arose. It would seem very unlikely, however, that the consistent immunogenicity of a class of neoplasms arises from an exposure of occult cell surface structures, and such an explanation is probably untenable in instances where the immunogenicity of tumors infected with the oncogenic viruses is shared by infected normal tissues.) Moreover, it is not unlikely that clones of cells passaged repeatedly in animals or in tissue culture may develop new (hence, antigenic) cell components as consequence of a very artificial existence, and not necessarily as consequence of their neoplastic state as such. Continued passage of cells undoubtedly also results in the selection of variants which possess characteristics favored by the conditions of the passaging, and these conditions may be very different from those prevailing when the cells are eventually examined immunologically. The discovery of immunological reactions directed by isogenic or autologous animals against neoplastic cells of very recent origin would therefore constitute much more convincing evidence for the neoplastic nature or association of the antigens than do similar findings obtained with old, albeit supposedly isogenic, tumors.

Accordingly, most of our experiments were conducted with newly arisen

tumors in the primary hosts, or with tumors in the first or second transplant generation in isogenic animals; tumors were never used after the eighth transplant generation.

It is obvious from the aforegoing discussion that a crucial condition for all tumor immunology experiments is the isogenicity of tumor donors and tumor hosts. However, complete homogeneity of characteristics controlled by multiple genetic loci on different chromosomes, as are the isoantigens of the mouse, is probably never attainable. The requirement for isogenicity is therefore formulated realistically as follows: differences in the composition of normal isoantigens between animals must be reduced to the point at which second set grafts of skin, a tissue very susceptible to immunological attack, are accepted permanently (or, for practical purposes, for at least four months) in a condition equivalent to that of autografts. To assure such a degree of isoantigenic similarity among our test animals, several precautions were adopted. Only animals with a carefully maintained pedigree were used. Reciprocal second set skin grafts were performed routinely on randomly selected pairs of animals of each strain studied, throughout the course of the experiments. With the exception of an occasional technical failure, these isografts were accepted as well as were autografts placed on each animal for control purposes. Pretreatment of the animals with a fraction of tubercle bacilli which stimulates immunological responsiveness (described below) failed to cause skin graft rejection. Intrastrain transplantation of a variety of tissues other than skin takes place routinely in the course of other studies carried on in our laboratories, and no indication of a heterozygosis of isoantigenic characteristics has come from these experiences. As a still further isogenicity control, parallel groups of animals were immunized in many of our experiments with normal tissues and were subsequently challenged with both skin grafts and tumor implants, and, conversely, animals immunized with tumors were challenged with skin as well as with tumor grafts. These controls similarly failed to reveal an operative heterozygosis of isoantigenic components. It is clear, therefore, that any specific acquisition of heightened reactivity against a tumor cannot be ascribed, in our experiments, to immunological reactions directed at *normal* tissue antigens not held in common by tumor host and tumor donor.

4. Effect on tumor resistance of a mycobacterial fraction which acts as an activator of immunological responsiveness

Our first experiments consisted of attempts to increase the resistance of mice to the development of mammary tumors *in situ* and to the growth of mammary tumor isografts by pretreatment with a material which behaves as a general activator of immunological responsiveness. The rationale of these experiments was based on the following consideration: if progressive neoplasia occurs only in animals suffering some degree of immunological disability, then correction of this disability by means of a stimulator of immunological responsiveness would be expected to increase resistance against the development of autochthonous

tumors and of tumor isografts. The material tested was a methanol-insoluble fraction of phenol killed, acetone washed attenuated tubercle bacilli of the BCG (Bacillus Calmette-Guerin) strain. This fraction, referred to as MER (methanol extraction residue), had been shown capable of heightening the resistance of experimental animals to infection with virulent tubercle bacilli [69], [70], [71], as well as with a large variety of antigenically unrelated microorganisms [72], [73], and had also been found to heighten antibody and homograft responsiveness, and the clearing activity of the reticuloendothelial system [51], [74].

The MER fraction was first tested for ability to heighten resistance to isografts of tumors of several different histological types, both spontaneous and induced. These experiments showed clearly that pretreatment with MER increases considerably the resistance of inbred mice against implants of many, but not all, isogenic tumors [75], [76]. The protective activity of the fraction was manifested against tumors of spontaneous origin as well as against those experimentally induced. Treatment begun *after* implantation of the tumors exerted a lesser, but in some instances still marked, protective effect.

The results obtained in further studies with spontaneous mammary carcinomas were similar: pretreatment with the MER heightened the resistance of mice to implants of these tumors, and also delayed the onset of indigenous, spontaneously developing neoplasms. The findings coming from two representative experiments are shown in figures 1 and 2.

As is seen from figure 1, pretreatment with MER inhibited markedly the development of the mammary tumor isografts into palpable tumors. The data presented in figure 2 illustrates the retardation of the appearance of indigenous tumors in the autochthonous hosts. In both experiments, treatment with the immunological activator took place a considerable period of time before experimental tumor challenge or spontaneous tumor onset; the long lasting protective effects elicited by this substance against microbial infection [73] are thus also manifested in the tumor systems. The protection evoked against the onset of spontaneous tumors was modest, but statistically significant. It must also be noted that in this experiment the protective activity of MER cannot possibly be attributed to an activation of immunological responses against residual isoantigenic impurities, and that artifacts inherent in transplantation experiments—surgical manipulation, the requirement for new vascular connections, and so forth—are avoided.

In studies of the MER in infectious disease systems it was noted that the, effective dose range is a very narrow one and that the optimum amount must be determined empirically for given experimental conditions [73]. An experiment was therefore designed in which C3H females were given two intraperitoneal injections of different quantities of MER at two and at five months of age, and were then observed for the development of spontaneous mammary tumors. There were 16 mice in each treatment group. A control group of animals was given saline only, and a fifth group was given an injection of living BCG organisms (0.5 cc of a 1:25 dilution of an eight day old culture in liquid medium).

Living BCG was tested even though our previous findings had indicated that MER is almost always a superior activator of immunological responsiveness [71], [73], [76], because of the reports of other workers that the living tubercle bacilli did increase resistance against some tumor homo- and isografts [77], [78]. The results of this experiment are shown in figure 3.

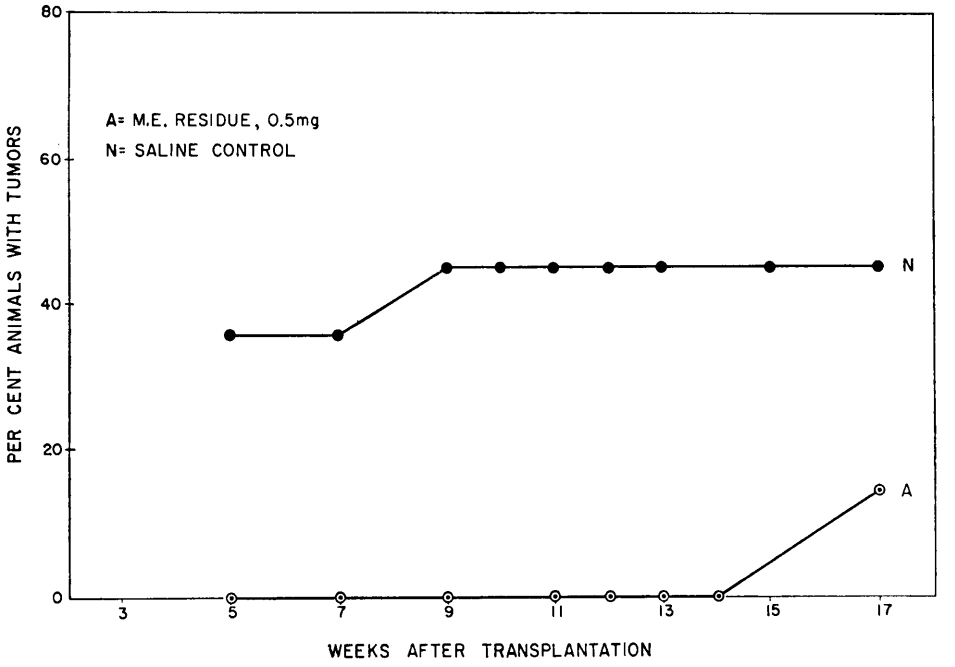


FIGURE 1

Effect of pretreatment with an immunological activator (MER) on the development of first transplant generation isografts of a spontaneous mammary carcinoma in RIII mice. (Data from [51].)

Young adult RIII females were given single intraperitoneal injections of 0.5 mg of MER in 0.5 cc saline, or of saline only. There were 15 mice in each group. Four months later, each animal was challenged with two subcutaneous implants of a newly arisen isogenic mammary carcinoma. (Each implant measured approximately 1.0 cubic mm.) The animals were then examined periodically for 17 weeks.

The protective effect of MER at a dosage level of 0.4 mg is clearly apparent from figure 3. Three times that quantity afforded little, if any, protection; and 3.6 mg, as well as the living BCG, elicited the opposite effect, acceleration of tumor development. As will be discussed in a succeeding section, an induced enhancement of tumor development does not contradict the operation of

immunological mechanisms, but the results do point to the importance of ascertaining the correct amount of MER for the induction of a predictable effect.

While these experiences with MER in the mammary tumor system strongly suggest the presence of immunological reactions against the tumors, it is con-

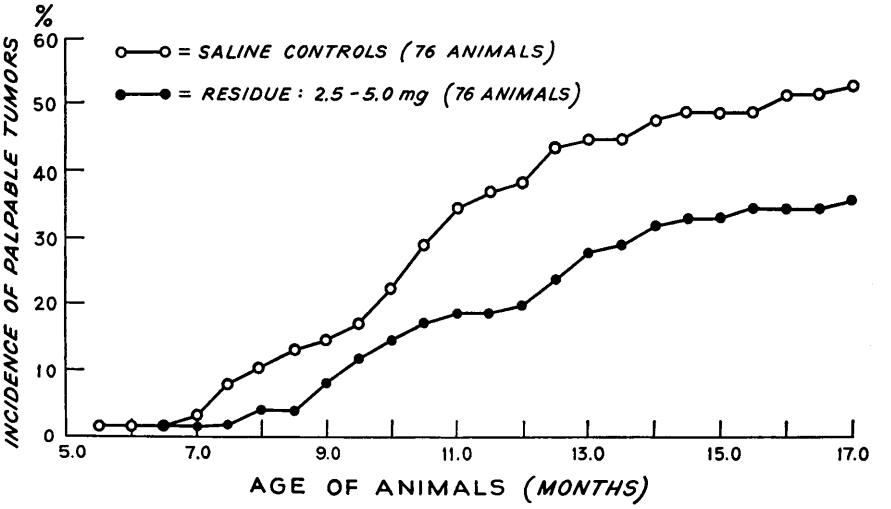


FIGURE 2

Effect of treatment of C3H mice with an immunological activator (MER) early in life on the spontaneous development of mammary carcinomas in late adulthood.
(Data from [51].)

C3H female mice were given several intraperitoneal injections of MER or of saline during the first 4 months of life, beginning at an age of 3 weeks. The total amount of MER given to each animal was between 2.5 and 5.0 mg. There were no obvious differences in the general health and breeding behavior between animals of the two groups. The animals were treated as normal breeding females, and the development of spontaneous tumors was followed by periodic inspection over a 17 month period.

ceivable that the tubercle bacillus moiety affected tumor resistance by means other than immunological: MER is a very impure preparation, and the possibility could not be ruled out entirely that one of its components exerted physiological effects not of immunological nature which caused the changed responses to the neoplasms. More direct evidence for the presence of tumor specific or tumor associated antigens of mammary tumors was required; the studies which provided this evidence are described in the following sections.

5. Heightened tumor reactivity in the primary host

The demonstration of heightened reactivity against a neoplasm in its primary host as result of a previous tumor experience offers stronger evidence for a

tumor specific reaction than do results accruing from experiments with isogenic animals; this has been discussed above and elsewhere [51]. On the other hand, the autochthonous tumor host constitutes a very difficult model in which to detect immunological processes: the host is often old; the debilitating initial

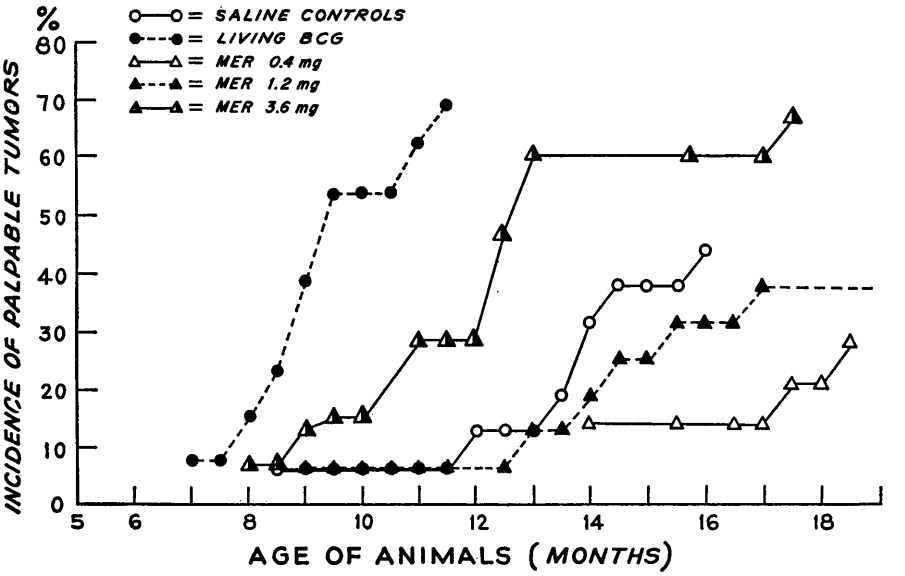


FIGURE 3

Effect of treatment of C3H mice with different quantities of MER and with living BCG on the spontaneous development of mammary carcinomas in late adulthood.

The animals received two intraperitoneal injections of living BCG or of different amounts of MER, at 2 and at 5 months of age. The total amounts of MER given in the two injections were 0.4, 1.2, or 3.6 mg. The animals were then placed into normal breeding routine, and were observed for the development of spontaneous mammary tumors for a period of 18 months.

tumor experience can be imagined to depress nonspecifically a variety of physiological functions including the immune response; the presence of the original tumor mass could well act as an antigenic depot, absorbing specific antibodies and specifically sensitized cells [79], or otherwise acting to initiate and maintain a state of specific "immunological unresponsiveness" [47] to [49], [80] to [83]; and the very occurrence of the spontaneous neoplasm might represent *prima facie* evidence for a degree of individual or strain determined immunological dyscrasia. We chose the autochthonous host as the original model for our study of specifically acquired reactivity against spontaneous mouse mammary carcinomas despite these difficulties, because it provides the most rigorous test of such reactivity.

Figures 4a and 4b illustrate the experimental designs employed in the initial studies. Newly arisen spontaneous mammary carcinomas were removed surgically from multiparous breeding females from our breeding colony. The tumors were maintained by freezing at −79°C or by single passage in an isogenic host.

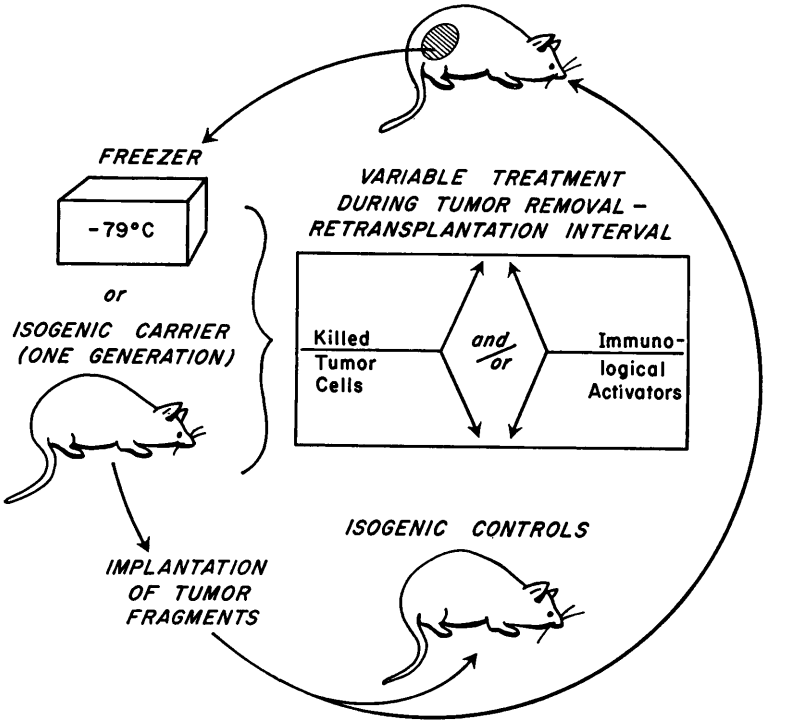


FIGURE 4a

Experimental design of studies of specifically acquired heightened reactivity against spontaneous mammary carcinomas in the primary autochthonous host: tumor reimplantation.

After intervals of time varying from experiment to experiment, small pieces of the tumor were transplanted back to the autochthonous host, and, at the same time, to groups of normal isogenic female mice of approximately the same age. In the interval between tumor removal and reimplantation, the autochthonous hosts, and, occasionally, some of the isogenic control animals, were given injections of tumor cell preparations killed by freezing and thawing and/or MER or another immunological activator (figure 4a). The rate of growth of the tumor implants was then followed, and several weeks after challenge the animals were sacrificed, their tumors weighed, and the draining lymph nodes examined.

An unexpected difficulty arose in the course of these experiments. Following removal of the initial mammary tumor, a large number of the animals rapidly developed new mammary tumors. These secondary neoplasms were not meta-

static foci of the original growth, but *bona fide* new cancers. It is not improbable that this occurrence is of immunological nature [84]; at any rate, many of the autochthonous animals were lost to the experiment. An additional source of tumor hosts was therefore developed in parallel studies, as schematized in figure 4b. An outgrowth line of preneoplastic tissue [58] arising in a C3Hf female (free of the MTV) was implanted into young females of another MTV

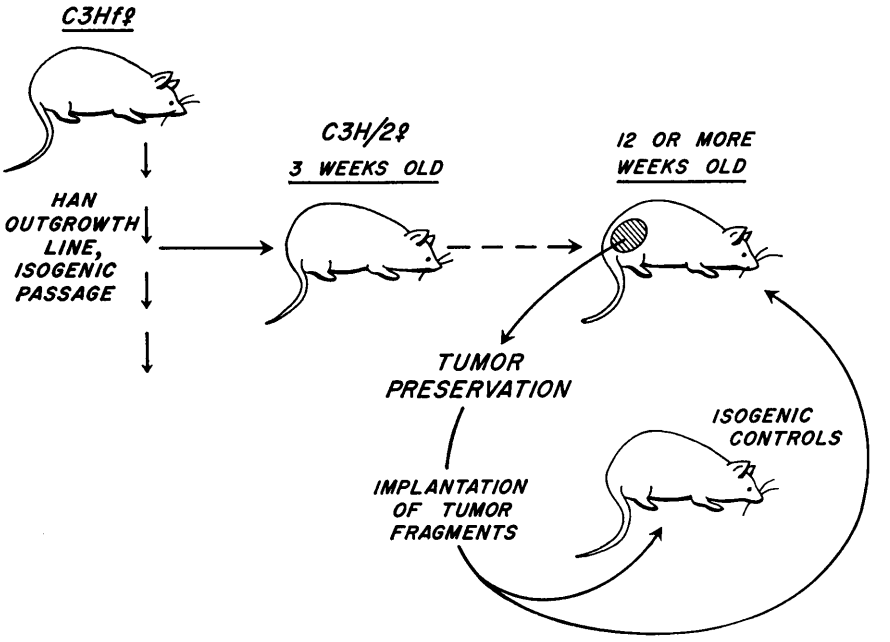


FIGURE 4b

Experimental design of studies of specifically heightened reactivity against spontaneous mammary carcinomas arising in young C3H/2 females from implants of C3Hf preneoplastic tissue outgrowth: tumor reimplantation.

free subline of the C3H strain, C3H/2. Although C3Hf nodules generally have a very low tumor potential, this particular outgrowth produced tumors in a large proportion of the animals. Following removal of these tumors (together with any remaining outgrowth tissue), no further tumors developed; this was not surprising in view of the fact that young C3H/2 mice have no, or only very few, preneoplastic nodules. The C3H/2 tumor hosts thus provided a ready population of primary tumor bearing animals with which the planned experiments could be performed. These hosts cannot be considered truly autochthonous, and are, accordingly, referred to as original tumor hosts; they still represent a more rigorous model for tumor immunity studies than do isogenic carriers of implanted tumors. (The designation f is appended to indicate that an animal, or a subline derived from such an animal, was foster nursed on a female of another strain. The

C3H strain, like many other strains of mice, is infected with the MTV. This agent is transmitted from generation to generation via the mother's milk. C3H animals foster nursed on females of an MTV free strain are therefore free of the virus, but are identical with C3H in genotype, and hence in isoantigenic makeup. The C3H/2 line is derived from a C3H female which lost the MTV spontaneously some generations ago; it, too, has remained isogenic with C3H. Both the C3Hf (sometimes written C3H/f) and the C3H/2 sublines are, however, infected with a related agent, recently termed NIV [85], which is transmitted through either ovum or sperm. NIV is capable of inducing preneoplastic nodules morphologically indistinguishable from those found in MTV infected strains, but most NIV induced nodules are considerably less tumorigenic.)

The results obtained in these studies have been described elsewhere [51], [84] and are here summarized only briefly. The autochthonous and original tumor hosts frequently displayed a degree of heightened reactivity towards their tumors upon second exposure, and the appearance of the lymphoid tissue draining the sites of tumor reimplantation suggested the occurrence of an active immunological response. The heightened reactivity of the animals was manifested either by increased resistance or increased susceptibility to the challenge tumor implants, relative to their growth in the isogenic hosts, and it seemed possible that both reactions were the manifestations of a specific immunological response. It is considered by most [86], [87], [88], though not by all [89], [90], present investigators that sensitized cells, rather than free circulating antibodies, play the dominant role in the rejection of most solid foreign tissues; conversely, it has been well established that circulating antibodies can act to *protect* a foreign graft against an otherwise effective immunological reaction—the phenomenon of immunological enhancement [91], [92]. Whether the immune response of an animal is largely in the direction of sensitized cells or of circulating antibody depends on a number of host and antigen dependent variables. The sensitivity of different tissues to immunological rejection also varies considerably, and it has been shown that immunological enhancement of one tissue and active rejection of another tissue of the same antigenic composition can occur simultaneously in an animal [93], [94]. It thus appeared quite possible that the immunological reactions initiated in some hosts against some tumors would be manifested by resistance, and in other instances by enhancement.

The analysis of results coming from experiments in which the test animals are the autochthonous or original tumor hosts poses some difficulty. No two spontaneous tumors can be assumed to be alike, and hence challenge of a primary tumor host and its controls with any one tumor represents one experiment. The experimental group in any one experiment is thus represented by a single animal. In some instances, a sufficient number of isogenic mice was available for challenge with a given tumor to give statistical significance to the comparison of the one experimental animal with its controls, but such comparisons are still unsatisfactory to the biologist. Another method of analysis was therefore employed. All the animals in each separate experiment were ranked according to

the amount of tumor growth which they permitted in a given period of time. The autochthonous or original hosts were then placed into percentile groupings based on their ranking, always relative to the controls challenged with their respective tumors, of degree of tumor support. It follows that the original and autochthonous tumor hosts would be distributed randomly into the several percentile groupings if their initial tumor experience had not endowed them with heightened reactivity against reexposure, that is, if the null hypothesis were to hold. A histogram constructed from such negative results would appear as indicated in figure 5a. In contrast, the histogram constructed from the *actual*

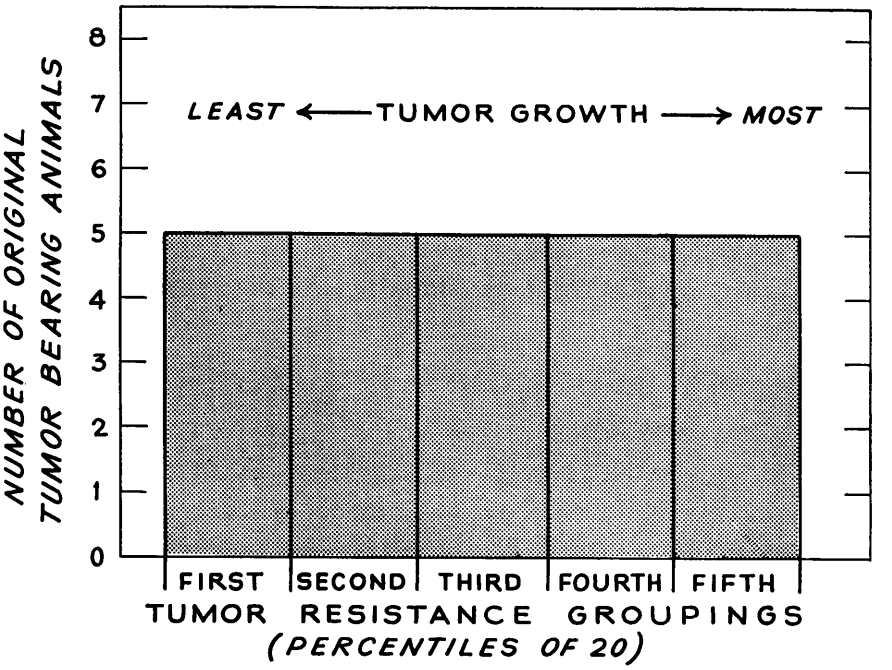


FIGURE 5a

Hypothetical distribution of original tumor bearing mice by rank of tumor resistance relative to the corresponding controls (in percentiles of 20), if the initial tumor experience had *not* evoked heightened reactivity, that is, *if the null hypothesis applies.*
See figure 5b for details regarding construction of the histogram.

results obtained in the first 21 experiments has the appearance shown in figure 5b. There was a marked skewing of the distribution of the original tumor hosts to the poles of least and most tumor growth relative to their own controls, that is, tumor resistance and tumor enhancement. These findings were corroborated in a series of further experiments based on the same design [51].

Although the primary host system possesses a number of distinct advantages

over those employing secondary isogenic tumor hosts, it still does not permit a true comparison with neoplasia in nature: the measure of heightened reactivity is the behavior of tumor *implants*, not that of the original neoplastic mass or of its metastatic foci. Moreover, the behavior of the challenge tumor implants in the original and autochthonous hosts can only be evaluated by *comparison* with the isogenic controls, and the potential danger of a residual lack of isogenicity remains inherent in this frame of reference [51]. Another test system was therefore constructed, as shown in figure 6.

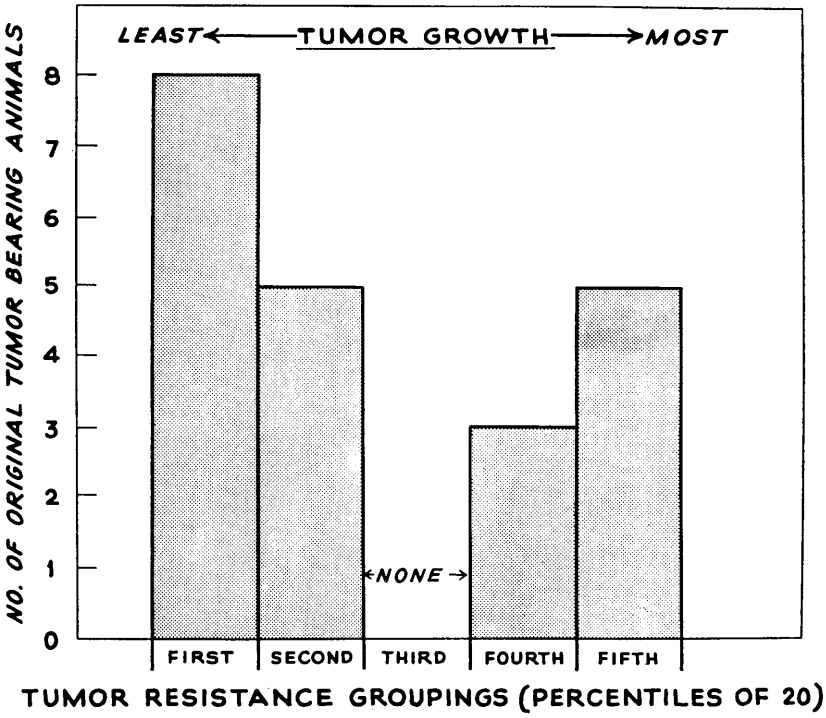


FIGURE 5b

Actual distribution of 21 original tumor bearing mice by rank of tumor resistance relative to their corresponding controls (in percentiles of 20). (Data from [84].)

The original C3H/2 host in each experiment is placed in one of five groupings of percentiles of 20, on the basis of its tumor resistance ranking relative to the corresponding isogenic control animals. The percentile groupings are arranged in decreasing order of tumor resistance of the original hosts. Thus, the first percentile group consists of original tumor hosts which ranked in the first fifth of their respective experimental groups, or better; the fifth percentile group contains original hosts which ranked in the last fifth, or lower; and the intermediate percentile groups contain original hosts whose relative rankings fell proportionately between the poles of resistance and enhancement.

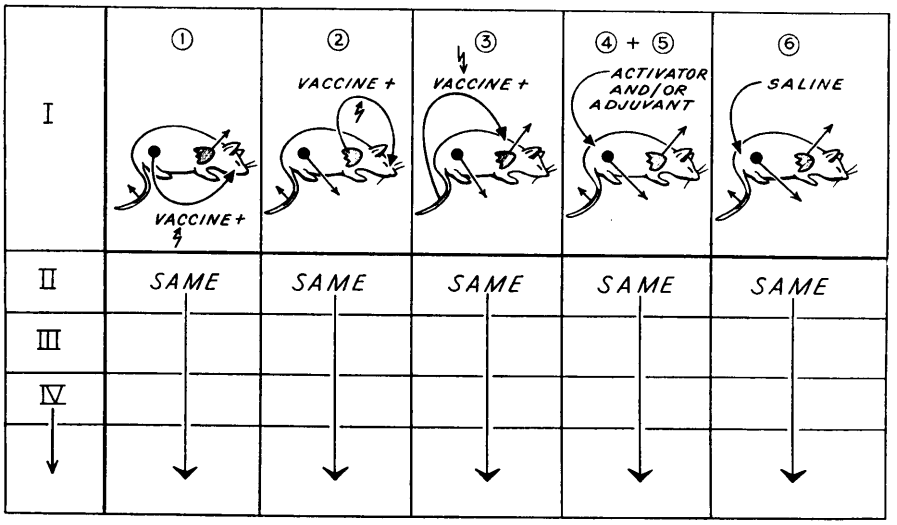


FIGURE 6

Experimental design of studies of specifically acquired heightened reactivity against spontaneous mammary carcinomas in the primary autochthonous hosts: tumors remaining *in situ*.

The growth of newly arisen tumors in groups of isogenic multiparous females is followed for a period of 7 to 12 days, and the animals are then subjected simultaneously to an identical operative procedure. A small piece of the tumor, of liver, and of tail skin are removed, with the bulk of the tumor mass remaining *in situ*. The tissues are homogenized and exposed to 15,000 r of X-irradiation, and the animals are immunized with a series of injections of *one or another* of these preparations, together with the MER activator and incomplete Freund's adjuvant [95]. Liver and skin preparations were employed, originally, as tissue controls. Other control animals, similarly operated, are given injections of MER, adjuvant, or saline only. The growth of the tumors after operation and treatment is again followed, and the mean growth rates of the differently treated groups are compared.

Contrary to original expectation, treatment with the autochthonous liver exerted a definite, though limited, retardation on further tumor development, whereas treatment with tumor tissue elicited only a marginal effect. Further controls were therefore added to the experiment, consisting of treatment with liver preparations from isogenic animals free of, or infected with, MTV. These experiments are still in progress, but the results obtained so far indicate that only liver from animals carrying MTV infected tumors is able to induce a significant tumor retarding effect (figure 7). A tentative interpretation of this finding, currently under test, is that tumor associated antigens may be present in an immunogenically activated state inside the phagocytic reticulo-endothelial cells

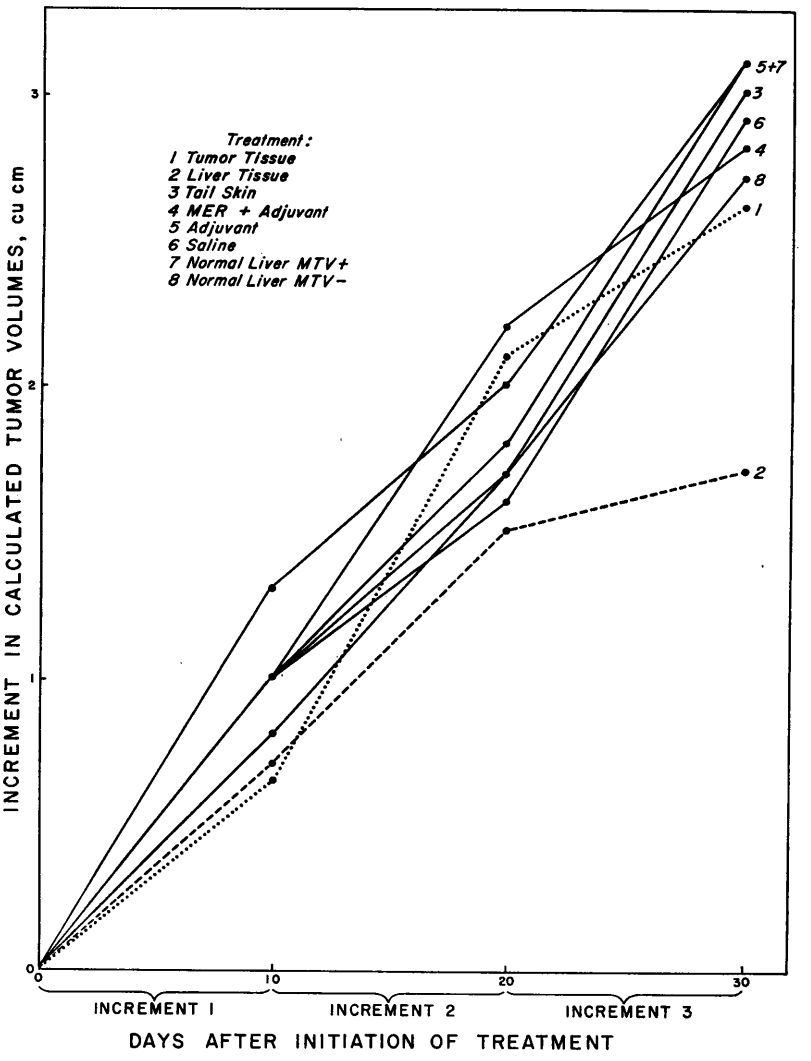


FIGURE 7

Effect of treatment with autochthonous liver and with other preparations on the development of spontaneous mammary tumors of C3H mice *in situ*. The figure shows increments in mean tumor size during three 10 day periods beginning with the initiation of treatment (15 to 20 mice per group). The animals were given a series of three injections, over a 12 day period, of autochthonous tumor, liver, or skin (lines 1 to 3); MER + adjuvant, adjuvant alone, or saline only (lines 4 to 6); or liver from normal, tumor free isogenic C3H (MTV+) or C3Hf (MTV−) mice (lines 7 and 8). (See Vaage and Weiss, to be published.) Tumor volumes are calculated from the equation $V = ab^2$ (0.4), where a is the larger and b the smaller of the two diameters bisecting the tumor evenly at right angles to each other; the derivation of this equation is discussed elsewhere [102].

of the liver; such an activation of the immunogenic capacity of antigens has recently been postulated by several workers [96], [97], [98].

Because it is unlikely that even a very active immunological reaction can affect significantly a large tumor mass in an old and debilitated animal, another series of experiments has been initiated, in which the original tumor is removed and the animals are given intravenous inoculations of viable cell suspensions of that tumor, to simulate metastatic spread. They are then treated as described in the preceding paragraphs, and are examined after a period of time for the presence and size of lung metastases. This study is still in progress.

The results already obtained from these several models employing the original or autochthonous tumor bearing animal as experimental host have thus provided definite indication of the operation of immunological mechanisms against mammary carcinomas of mice. However, these models suffer from the major drawback that the evaluation of results must be based on comparisons of *populations* of such animals which had received different treatment, or on comparisons with isogenic populations challenged with the corresponding tumors. The behavior of any one animal towards its tumor is obscured in such analyses. Moreover, it is impossible to vary the experimental conditions for the testing of any one neoplasm, and it is impossible to ascertain whether the outcome of a given experiment reflects largely host or tumor peculiarities. Once having established the existence of an immunology of spontaneous mouse mammary carcinomas in these awkward primary host systems, we therefore undertook further studies with isogenic animals, sacrificing some of the advantages inherent in the earlier models, but gaining the ability to investigate more systematically the extent and nature of the tumor directed immunological reactions.

6. Heightened tumor reactivity in isogenic hosts

A large number of experiments have been conducted in several strains of mice during the past four years, employing numerous spontaneous mammary tumors, newly arisen or within the first few transplant generations, and utilizing the experimental outline depicted in figure 8. Comparable, randomly distributed groups of isogenic animals are immunized with a living tumor graft or with one of several kinds of normal tissue. When the tumors reach a size of approximately 50 to 200 cu mm they are removed surgically; simultaneously, the normal tissue implants are removed from the control animals. In some experiments, the animals receive further immunization with preparations of inactivated tumor or normal tissues, and in others treatment with such killed tissue preparations takes the place of a first experience with living tumor. Groups of animals immunized with the neoplastic and the normal tissues are subsequently challenged with a second implant of the tumor, or with normal mammary tissue or skin from the original tumor host or from an isogenic animal. Both immunization and challenge with tumor tissue are thus controlled by parallel exposure of the control animals to normal tissue.

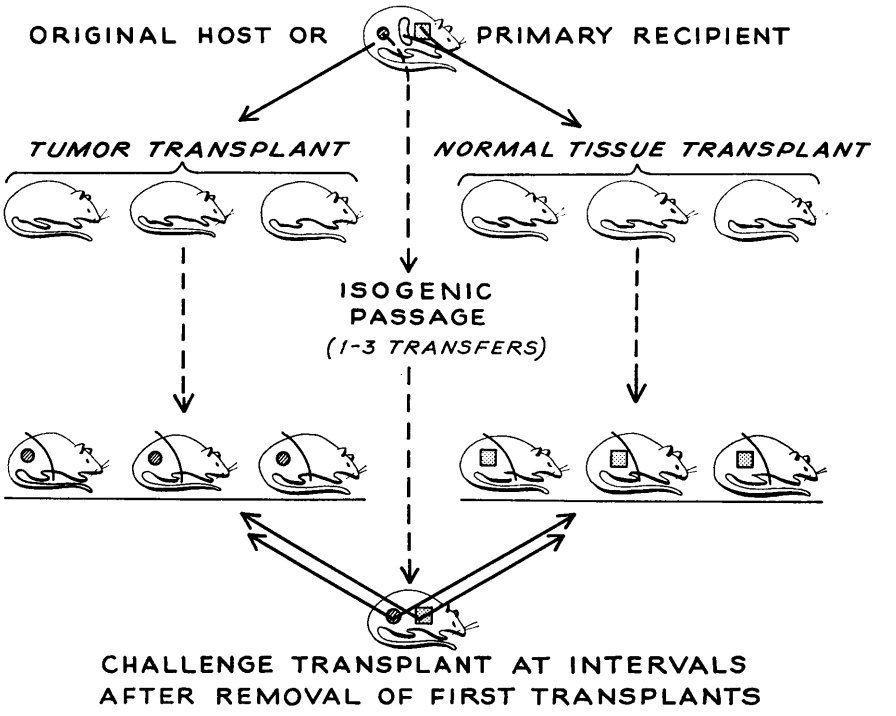


FIGURE 8

Experimental design of studies of specifically acquired heightened reactivity against spontaneous mammary carcinomas in isogenic hosts.

The results of these experiments are reported in detail in a number of recent communications [99] to [103], and they provide further clear evidence that immunization with neoplastic mammary tissue evokes a state of heightened resistance, and, occasionally, a state of heightened susceptibility, to a subsequent challenge with that tumor. In none of these experiments was there any indication of failure of the animals to accept grafts of skin or other normal tissues, regardless of whether they had been previously immunized with normal or neoplastic tissue, or whether they had received no initial treatment; neither were tumor implants rejected or retarded in their development as result of a first experience with normal tissue.

The results of a typical experiment are presented in table I. It is evident from table I that immunization with living tissue of a rapidly growing tumor, followed by further treatment with X-irradiated tumor cells, conferred complete protection on 2/3 of the animals against challenge with tumor implants sufficiently "virulent" to produce progressively growing neoplasms in all of the controls. The protection elicited by similar immunization with a slowly growing tumor was of a much more modest order, manifested only by a retardation of

TABLE I

DEVELOPMENT OF GRAFTS OF A FAST AND A SLOW GROWING C3H SPONTANEOUS MAMMARY CARCINOMA IN ISOGENIC C3H/2 HOSTS PRETREATED WITH LIVING AND X-IRRADIATED TUMOR OR NORMAL TISSUES
(Data from [99].)
The experiment was terminated after 60 days.

Immunization	Animals with Tumors	Positive Implants
	Total	Total
	Fast Growing Tumor	
Living tumor graft, irradiated tumor cells ("test")	8/22	13/44
Living mammary graft, irradiated liver and kidney cells ("control")	12/12	22/24
	Slow Growing Tumor	
Test	9/9	18/18
Control	8/8	16/16
	Avg. Time of Appearance	Avg. Size at Termination
Test	35 days	0.7 cu cm
Control	22 days	1.6 cu cm

the development of the challenge implants. The greater immunogenicity of rapidly developing mammary tumors appears to be a general phenomenon, and may be related to the finding of Prehn [104] and Old, Boyse, Clarke, and Carswell [105] that sarcomas of mice arising rapidly after application of methylcholanthrene are more antigenic than are those appearing after prolonged periods of time; the possible reasons for the association of rapidity of growth with marked antigenicity are discussed in another communication [99].

It remained to be shown that the heightened resistance against mammary tumors which accrued from an initial, experimentally terminated experience with the neoplasms was indeed of immunological nature (that is, brought about by circulating antibodies or specifically sensitized cells). This was demonstrated in further experiments which revealed that the heightened tumor resistance could, in fact, be transferred to normal mice by means of living lymphoid cells taken from immunized isogenic donors (that is, passive transfer of immunological reactivity). Such an experiment is illustrated in table II.

It is seen from table II that increased tumor resistance could be bestowed on normal mice by means of lymph node and peritoneal washing cells derived from a tumor immunized donor, but not by means of such cells from a normal animal, nor with small quantities of either normal or immune serum. These findings are

TABLE II

PASSIVE TRANSFER OF SPECIFICALLY ACQUIRED TUMOR RESISTANCE BY MEANS OF IMMUNE LYMPHOID CELLS
(Data from [99].)

Young adult C3Hf mice were challenged subcutaneously with a suspension of living cells of a C3H spontaneous mammary carcinoma 1 or 7 days (the data are here combined) after receiving serum or lymphoid cells taken from tumor immunized or normal C3H/2 animals. Tumor challenge: 2×10^5 cells, SC; serum: 0.2 cc, IP; donor cells: 2×10^6, IP.

Donor Material	No. Animals with Tumor/Total
Immune serum	6/6
Normal serum	6/7
Immune peritoneal washing cells	6/16
Normal peritoneal washing cells	14/15
Immune lymph node cells	2/8
Normal lymph node cells	8/8
None	7/8

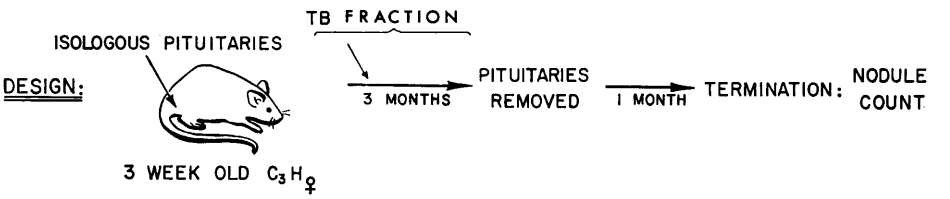
in accord with the classic observations on the passive transfer of specifically acquired resistance against tissues of known, foreign antigenic composition, and make it appear most unlikely that the heightened tumor resistance of the immunized animals accrued from some nonimmunological changes induced by the initial tumor experience.

The next phase of these studies was to determine *when* in the progression of changes leading from normal mammary parenchyma to the preneoplastic hyperplastic alveolar nodule, and eventually to the frank mammary carcinoma, the antigens responsible for the immunogenicity of the tumors arise. These experiments, in turn, provided information towards an understanding of the *biological nature* of the protective antigen(s).

7. Studies on the nature of the protective antigens

7.1. *Experiments with* C3H *and* C3Hf *mice.* The first experiments (conducted in collaboration with Dr. Howard Bern and Miss Donna Brown) designed to determine at what point in the transformation of normal mammary tissue to carcinoma there arise the antigens responsible for the specific immunogenicity manifested by the neoplastic cells again took advantage of the ability of the MER fraction of tubercle bacilli to activate immunological responsiveness. As has been described above, treatment with this material was found to heighten the reactivity of inbred mice against tumors. The question now asked was whether administration of MER could elicit a similar effect against the preneoplastic tissue.

The experimental design, and the results obtained in the first trial [51], are depicted in figure 9. Young C3H females received intramammary implants of



RESULTS: TREATED: 12 MICE: 0,0,0,0,0,0,0,0,0,0,6,13 = 19 NODULES /2 ANIMALS
CONTROL: 10 MICE: 0,0,1,1,6,11,13,33,34,71 = 170 NODULES /8 ANIMALS

FIGURE 9

Effect of the MER tubercle bacillus fraction on the development of the preneoplastic hyperplastic alveolar nodules in the mammary glands of C3H mice. (Data from [51].)

three or four pituitaries, derived from newborn isogenic donors, to stimulate lobular alveolar development of their mammary glands. During this period of time, the animals in one group received an intraperitoneal injection of saline, the animals in the other group 0.5 mg of MER. The pituitary implants were removed after three months. Following an interval of one month thereafter (to allow the stimulated mammary glands to again regress), the animals were sacrificed, and the number of nodules in the fixed and stained mammary gland preparations were counted. As seen from figure 9, treatment with MER resulted in the appearance of considerably fewer preneoplastic nodules.

This experiment was repeated, with results in the same direction [106]. Of 26 C3H female mice stimulated to lobular alveolar development by means of pituitary implants or hormone injections (estrogen and deoxycorticosterone) and treated with saline only, 17 animals were found to possess preneoplastic nodules; in contrast, only 12 of 30 animals similarly stimulated but given MER showed nodular structures ($P = <0.1, >0.05$). The mean number of nodules in the nodule containing control mice was 5.3, that among the MER treated group 2.7; this difference was statistically significant.

These findings suggested that preneoplastic as well as neoplastic mammary tissue possesses antigen(s) which distinguishes it from normal tissue, but, as discussed in relation to the activity of MER against tumors, the possibility that the tubercle bacillus material affects the development of a particular tissue for nonimmunological reasons cannot be excluded. Direct immunization experiments with the nodular tissue were therefore initiated.

The results of these experiments have recently been published [100], [101]

and they have provided strong evidence for a major role of the MTV in the antigenicity of spontaneous mammary tumors and hyperplastic alveolar nodules. These findings can be summarized as follows.

(1) HAN outgrowth tissue derived from a C3H female (MTV+) grew well in both young (that is, immunologically not fully mature) and adult C3H hosts, but developed poorly in young isogenic C3Hf animals (MTV−), and not at all in C3Hf adults (figure 10, table III). In contrast, implants of C3Hf nodular outgrowth grew without obvious impediment in both C3H and C3Hf recipients.

(2) Immunization of adult C3Hf mice with C3H nodular outgrowth bestowed on them a marked degree of resistance against later challenge with tumor implants derived from that outgrowth (table IV). On the other hand, immunization of adult C3Hf mice with C3Hf derived nodular outgrowth did not afford clear protection against challenge with a tumor which had developed from *this* outgrowth tissue (table V).

(3) Immunization of three week old (that is, immunologically not yet fully mature) C3Hf hosts with C3H nodular tissue did not result in appreciably heightened tumor resistance (table VI).

(4) Early transplant generation implants of mammary tumors of C3H origin developed in considerably fewer adult C3Hf than C3H hosts, regardless of whether or not the former were previously immunized with MTV containing tissue (table VI, table VII). This finding corroborates the observation recorded some years ago by Barrett, Deringer, and Dunn that mammary tumor isografts progress more rapidly in mice infected with MTV than in mice of the same genotype free of the virus [107].

(5) To be certain that the immunogenicity of C3H tissues in C3Hf hosts did not arise from a hidden residual heterozygosis of isoantigenic composition between the two lines, the reaction of C3Hf mice to preneoplastic and neoplastic C3H mammary tissues was studied comparatively in the MTV free animals and in C3Hf animals reinfected with MTV at birth by foster nursing on a C3H mother. It is seen from table VIII that infection with MTV at birth rendered the C3Hf mice as unresponsive to mammary nodules and tumors as the C3H animals. Infection with MTV, rather than an occult difference in composition of normal tissue antigens between the C3H and C3Hf sublines was thus seen to be the significant determinant in the distinctive immunizability of the latter. It was also shown that infection with MTV did not lower immunological responsiveness in a *general manner:* infected animals responded as well as uninfected ones to challenge with an MTV free methylcholanthrene induced mammary carcinoma, and to immunization with sheep red blood cells and bovine serum albumin [108].

It is apparent from this series of observations that at least one category of antigen(s) responsible for the immunogenicity of spontaneous mammary carcinomas of mice is dependent on the presence of the MTV. Different functions of the MTV in this immunogenicity can be visualized. The antigen(s) could be the virion *per se*, or a fragment thereof; it could be a host cell product, coded

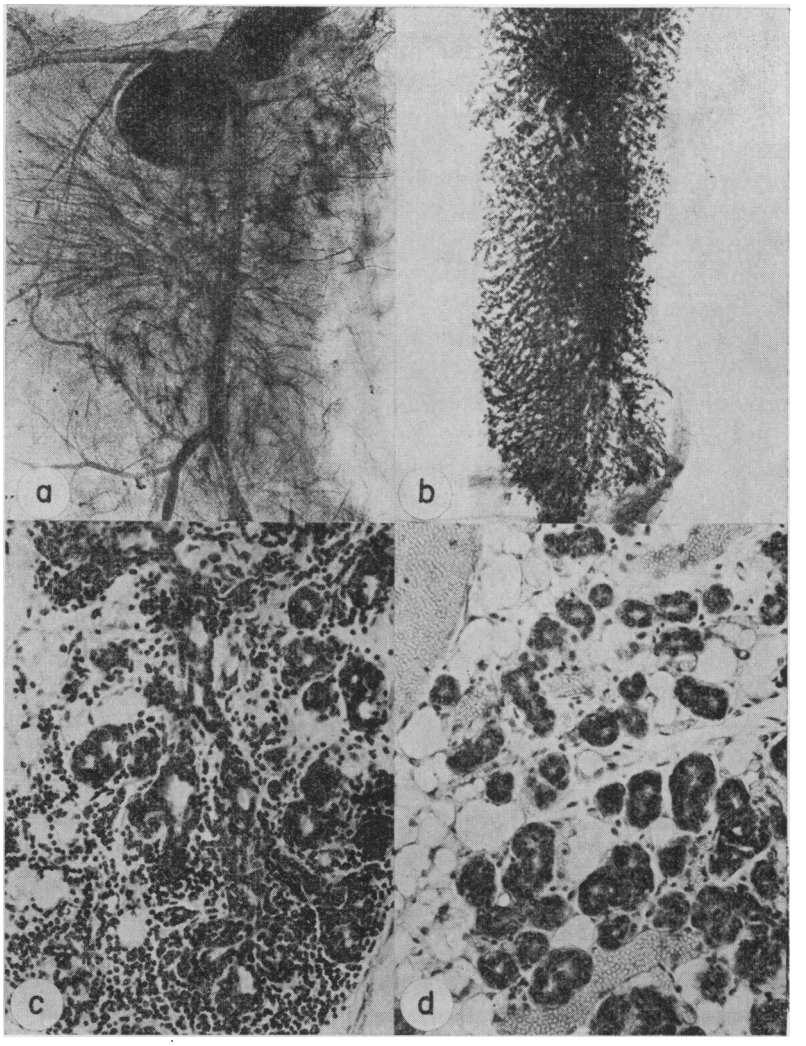


FIGURE 10

Appearance of a C3H nodular outgrowth in young (that is, immunologically not fully mature) C3H and C3Hf hosts.

(a) A C3H nodular outgrowth in the inguinal fat pad of a 15 week old C3Hf virgin female. The implant was introduced when the animal was 3 weeks old. Note, sparse, filamentous development of the implanted tissue. Stained with iron hematoxylin, × 9.

(b) Same C3H nodular outgrowth in the inguinal fat pad of a 13 week old C3H virgin female. The implant was introduced at 3 weeks of age. Stained with iron hematoxylin, × 7.5.

(c) Histologic section of the C3H nodular outgrowth taken from the C3Hf host

TABLE III

DEVELOPMENT OF A C3H NODULAR OUTGROWTH IN THE MAMMARY FAT PADS OF YOUNG AND ADULT C3H AND C3Hf HOSTS

(Three week old mice are not fully mature immunologically.)
Figures in parentheses show number of mammary fat pads examined after 10 weeks of growth.

Age of Mice in weeks	Fat Pad Area Occupied (mm^2) in Mice of Strain			
	C3H(MTV+)		C3Hf(MTV−)	
3	41	(14)	22	(12)
10 to 15	50	(6)	—	
30 to 36	23	(12)	1	(9)

for by the viral genome; or it could be a host cell substance coded for by the host genome, with the virus acting as a specific derepressor (a model for this possibility is afforded by the recent work of Joklik with pox virus [109], [110]). The inability of mice infected with MTV at birth to respond readily to MTV

TABLE IV

EFFECT OF A PREVIOUS EXPERIENCE (THAT IS, IMMUNIZATION) WITH EITHER NODULAR TISSUE OF C3H ORIGIN OR NORMAL MAMMARY TISSUE OF C3Hf ORIGIN ON THE RESISTANCE OF C3Hf MICE AGAINST ISOGRAFTS OF A SPONTANEOUS MAMMARY CARCINOMA ARISEN FROM THAT NODULAR OUTGROWTH

Animals: C3H/f ♀ ♀ ; *nodule and tumor:* C3H;
schedule: nodule exposure, week 17–26; tumor challenge, week 28.

Previous Experience with	Number of Animals with Tumors/ Total at Following Times after Tumor Challenge (days)					
	7	15	18	22	37	67
Nodule	0/26	0/26	3/26	5/26	7/26	7/26
Normal Mammary Tissue	0/25	11/25	14/25	16/25	18/25	18/25

infected preneoplastic and neoplastic mammary tissues can be accounted for tentatively by assuming a state of complete or partial immunological unresponsiveness to antigen(s) dependent on the presence of the virus, and expressed soon after infection. Such specific immunological unresponsiveness is most

at 15 weeks of age. Note marked cellular infiltration. Stained with hematoxylin and eosin, × 250.

(d) Histologic section of the C3H nodular outgrowth taken from the C3H host at 13 weeks of age. Note absence of cellular reaction. Stained with hematoxylin and eosin, × 250.

(Data from [100].)

TABLE V

EFFECT OF A PREVIOUS EXPERIENCE WITH EITHER NODULAR OR NORMAL MAMMARY TISSUE OF C3Hf ORIGIN ON THE RESISTANCE OF C3Hf MICE AGAINST ISOGRAFTS OF A SPONTANEOUS MAMMARY CARCINOMA ARISEN FROM THAT NODULAR OUTGROWTH

Animals: C3H/f ♀ ♀; *nodule and tumors:* C3H/f;
schedule: nodule exposure, week 17–28; tumor challenge, week 31.

Previous Experience with	Number of Animals with Tumors/ Total Animals at Following Times after Tumor Challenge (weeks)					
	6	8	10	12	14	16
Nodule	15/30	16/30	16/30	17/30	20/29	21/28
Normal Mammary Tissue	16/27	15/26	15/25	15/24	18/24	18/22

readily induced by exposure early in life to antigens which are maintained in the tissues [111], [112]; specific unresponsiveness to microbial antigens has been demonstrated by a number of investigators [113] to [116]; and the occurrence of such unresponsiveness in nature has been shown in another viral system of mice, endemic lymphocytic choriomeningitis [117], [118], [119].

The inability of C3Hf HAN to induce heightened reactivity against C3Hf tumors is also based, presumably, on a state of specific immunological unresponsiveness to any antigen which might be controlled by the NIV. The observation that an initial experience with a C3Hf *tumor* may result in resistance or enhancement [84] does not necessarily constitute contrary evidence. Antigen(s) in-

TABLE VI

DEVELOPMENT OF ISOGRAFTS OF A SPONTANEOUS C3H MAMMARY CARCINOMA IN C3H AND C3Hf HOSTS IMMUNIZED WITH A C3H NODULAR OUTGROWTH AT DIFFERENT AGES

Tumor size: large means greater than 15 × 15 mm (no host resistance);
small means less than 10 × 10 mm (host resistance).
Experiment terminated 11–12 weeks after tumor challenge.

Age at Nodule Implantation	Age at Tumor Challenge	C3H Animals		C3H/f Animals	
		Tumor Incidence	Tumor Size	Tumor Incidence	Tumor Size
3 weeks	10–15 weeks	19/21	Large	16/21	Large
11 weeks	23–28 weeks	—	—	3/7	Large
30–36 weeks	37–43 weeks	17/19	Large	0/9	None
		36/40			
No nodule pretreatment (controls)	8–16 weeks (C3H/f) 15–36 weeks (C3H)	24/25	Large	12/18	Small

TABLE VII

DEVELOPMENT OF ISOGRAFTS OF A SPONTANEOUS C3H MAMMARY CARCINOMA IN YOUNG ADULT (THAT IS, IMMUNOLOGICALLY MATURE) C3H AND C3Hf HOSTS

Young adult C3Hf mice were immunized repeatedly with high speed centrifugation pellet preparations [15] of lactating mammary glands derived from mice of BALB/c and C3H genotype with and without the MTV. They were challenged 11 days after the last vaccine injection, later with a subcutaneous implant of a C3H mammary tumor. It is seen that, regardless of the nature of the immunization, only a small proportion of the C3Hf mice develop tumors, whereas all but one of 12 normal C3H controls supported progressive tumor growth. (Lavrin and Weiss, to be published.)

The difference between adult C3H and C3Hf hosts seen in this experiment is greater than that found in the experiment depicted in table VI, where 12 of 18 unimmunized C3Hf mice supported growth of the challenge tumor grafts, as against 24 of 25 C3H animals. The difference between these experiments can probably be ascribed to the fact that different tumors, presumably possessing to different degrees the ability to establish themselves in MTV free hosts, were used for challenge; other explanations have also been advanced [108].

Strain	Immunization with	Number of Animals	Number of Animals Developing Tumors upon Challenge with Tumor Isografts
C3Hf	BALB/c lactating gland	21	6
C3Hf	BALB/cfC3H lactating gland	22	5
C3Hf	C3Hf lactating gland	22	4
C3Hf	C3H lactating gland	22	7
	Total	87	22
C3H	none	12	11

dependent of the presence of the oncogenic virus might be expressed more strongly in neoplastic than in preneoplastic tissue; and, as will be discussed below, experience with a growing tumor can be imagined more readily to "break" a state of specific unresponsiveness.

If the inability, or lesser ability, of MTV infected mice to respond immunologically to spontaneous mammary neoplasms is indeed accounted for by a degree of specific immunological unresponsiveness (as has also been postulated by Morton in explanation of similar findings obtained in studies with MTV(+) and MTV(−) reciprocal F_1 hybrids [63], [64], [65]), then the failure of several other investigators to demonstrate the immunogenicity of these tumors finds a ready explanation: they attempted the difficult task of demonstrating immune reactions in MTV infected, and hence at least partially unresponsive, animals.

7.2. *Experiments with* BALB/c *and* BALB/cfC3H *mice.* It is not essential to our postulation of a specific immunological unresponsiveness to MTV associated antigens in MTV infected mice that these antigens are, in fact, expressed immediately after neonatal infection. Immunological unresponsiveness can be induced

TABLE VIII

DEVELOPMENT OF A C3H NODULAR OUTGROWTH AND OF A TUMOR ARISEN THEREFROM IN YOUNG ADULT C3Hf MICE (MTV−) AND C3Hf MICE FOSTER NURSED FROM BIRTH ON A C3H MOTHER (C3H/f/f.C3H) AND HENCE MTV+ (Data from [101].)

The animals received implants of a C3H nodular outgrowth into cleared mammary fat pads at 15 weeks of age. The fat pads were removed six weeks later, and the amount of nodular growth was ascertained. Two weeks thereafter, the animals were challenged with subcutaneous implants of a mammary tumor arisen from that outgrowth. The experiment was terminated 10 weeks after tumor challenge.

	Nodule Growth		Tumor Growth	
Host Animals	Nodule Incidence (>1 mm²)	Average Nodule Size (mm²)	Tumor Incidence	Tumor Size Range
C3H/f (MTV−)	3/13	0.5 (26 pads)	5/13	2 × 2 − 6 × 6 mm median 3 × 3 mm
C3H/f/f.C3H (MTV+)	10/11	49 (18 pads)	10/10	5 × 5 − 30 × 23 mm median 16 × 14 mm

in *adult* life with large amounts of antigen [81], [82], [83], and it could be imagined that the MTV associated antigen(s) detected in preneoplastic and neoplastic mammary tissue comes into being only some time after the virus reaches mammary tissue. Demonstration of a common MTV associated antigenicity of normal mammary tissue of infected young adults and of preneoplastic and neoplastic mammary parenchyma would point most convincingly to a major role of the virus in the immunogenicity of the tumors. Accordingly, experiments were conducted to determine whether immunization with preparations of normal, lactating mammary tissue, taken from young mice following their first pregnancy (and thus prior to the appearance of the preneoplastic structures) could protect against later challenge with tumor isografts. The results [108] can be summarized as follows.

(1) Immunization of young adult (that is, immunologically mature) BALB/c mice (MTV free) with normal lactating gland preparations of BALB/cfC3H (hence, MTV infected), but not of BALB/c, origin gave significant protection against subsequent challenge with BALB/cfC3H tumors (table IX).

(2) Unimmunized young adult BALB/cfC3H animals permitted more rapid growth of tumor isografts than did normal, uninfected, isogenic BALB/c animals (table X).

7.3. *Cross immunogenicity in the* C3H-C3Hf *system.* Another series of experiments has been initiated to provide still further support for the role of the MTV in the immunogenicity of these neoplasms. These experiments are designed to determine whether there exists an appreciable cross reactivity among spontaneous mammary carcinomas of mice, and whether immunization with MTV infected normal or preneoplastic tissue of *allogeneic* (that is, antigenically

TABLE IX

DEVELOPMENT OF ISOGRAFTS OF A BALB/cfC3H SPONTANEOUS MAMMARY CARCINOMA IN YOUNG ADULT BALB/c MICE IMMUNIZED WITH PREPARATIONS OF LACTATING MAMMARY GLAND DERIVED FROM BALB/c (MTV−) AND BALB/cfC3H (MTV+) DONORS
(Data from [108].)

Young adult BALB/c mice were given four intraperitoneal injections (over a period of one month) of tissue homogenates derived from BALB/c or from BALB/cfC3H normal lactating glands. Four weeks after the last injection, the animals were challenged subcutaneously with a suspension of living tumor cells. There were 20 BALB/c females (11 weeks old) per group. *Challenge dose:* 4×10^4 tumor cells. Tumor in first transplant generation.

Immunization with Normal Mammary Tissue	Cumulative Number of Palpable Tumors at Following Times after Challenge (days)					
	20	28	35	46	53	63
MTV (+)	3	6	9	10	10	10
MTV (−)	7	12	15	16	17	18

foreign) origin can evoke heightened reactivity against challenge with isogenic implants of HAN or neoplastic tissues. The basis for this approach is that the mammary tumor *viruses* isolated from different strains of infected mice share many common characteristics, although they do not appear to be identical [120], [121]; and it is known that other tumors initiated by the same or by related oncogenic viruses cross react immunologically [122].

TABLE X

DEVELOPMENT OF ISOGRAFTS OF A BALB/cfC3H SPONTANEOUS MAMMARY CARCINOMA IN NORMAL YOUNG ADULT BALB/c (MTV−) AND BALB/cfC3H (MTV+) MICE
(Date from [108].)

Challenge dose: 1×10^5 tumor cells. Tumor in 8th transplant generation.

Mice (females 20 weeks old)	Mean Tumor Volumes (mm^3) at Following Times after Challenge (weeks)		Number of Animals Dead with Large Tumors at Following Times after Challenge (weeks)		
	2	3	4	5	6
BALB/c	10	140	0/13	1/13	3/13
BALB/c/fC3H	20	280	5/11	7/11	8/11

The results obtained to date in these studies suggest that mice *can* be immunized against tumor isografts by means of allogeneic, MTV infected mammary tissue [108], [123], and that there is considerable cross immunogenicity of MTV infected tumors in MTV free hosts [103]; the latter finding is illustrated in table XI.

7.4. *Experiments with* C3H *and* C3H/2 *mice.* The results described in the preceding paragraphs have come from studies conducted in two systems: C3H-

TABLE XI

CROSS IMMUNOGENICITY OF C3H SPONTANEOUS MAMMARY CARCINOMAS IN C3Hf MICE

Young adult C3Hf mice were immunized either with implants of C3Hf normal tail skin or with one of several newly arisen C3H mammary tumors. The immunizing implants were placed subcutaneously into both rear legs. Twenty days after implantation, the hind legs were amputated at the shank. Fifteen days thereafter, the animals were challenged with the corresponding, and with the other, tumors. Challenge was with 1×10^4 viable tumor cells, placed subcutaneously into each inguinal area. At the time of preparation of this table, seven weeks had elapsed after challenge. (Shen and Weiss, to be published)

Immunization with	Challenge with (C3H tumor)	Number of C3Hf Mice with Tumors 7 Weeks after Challenge
C3Hf normal tail skin	#3	9/9
C3H tumor #3	#3	1/7
C3H tumor #4	#3	3/10
C3H tumor #5	#3	2/10
C3Hf normal tail skin	#4	9/10
C3H tumor #3	#4	1/10
C3H tumor #4	#4	1/10
C3H tumor #5	#4	2/10
C3Hf normal tail skin	#5	9/10
C3H tumor #3	#5	2/9
C3H tumor #4	#5	2/10
C3H tumor #5	#5	1/9

C3Hf, and BALB/c-BALBcfC3H. Support for the general applicability of the hypothesis deduced from these observations has come recently from the very similar results obtained by Dr. David Lavrin in identical studies initiated in our laboratory in a third system: immunization of the other MTV free C3H subline, C3H/2, with MTV infected C3H mammary tissues [124].

7.5. *Experiments in strain* A *mice.* A question which now presented itself was whether the MTV associated antigen(s) of mouse mammary tissues represent the *only* class of antigen underlying the immunogenicity of spontaneous mammary tumors, or whether there exist as well other antigens, not dependent on the presence or the biological activities of the MTV.

A consideration of the data already presented in this communication seems to give contradictory evidence. On the one hand, the demonstration of a heightened, albeit limited, reactivity of MTV infected autochthonous tumor hosts against a second tumor experience, and the similar heightened reactivity of the "original" C3H/2 tumor hosts *vis-à-vis* their tumors (MTV free, but NIV positive), points to the immunogenicity of the neoplasms in hosts of the same viral status, and therefore, presumably, to a category of antigens independent of the MTV or NIV. On the other hand, the apparent inability to demonstrate such virus independent immunogenicity in *isogenic* C3H hosts appears to argue to the contrary. There is, however, a considerable body of evidence coming from

parallel studies carried out in another strain of mice, the A strain, which shows that significant degrees of heightened resistance and heightened susceptibility to tumor challenge *can* be evoked in animals of identical genetic and viral composition.

The strain A mice maintained in our breeding colony are infected with the MTV, and the incidence of spontaneous mammary tumors among multiparous females is of the same order as that in C3H mice. However, the mean time of appearance of the tumors is significantly later in multiparous strain A females; and the tumor incidence in virgins is significantly lower than in the C3H strain [121]. This behavior of strain A mice is compatible with other observations which suggest that this strain may be more reactive immunologically than are other strains of mice [125] to [128]. Extensive immunological studies were accordingly conducted in this strain, in the expectation that antitumor responses might be more readily demonstrable [51], [102], [129]. The findings can be summarized as follows.

(1) Immunization of young adult strain A mice with *either* isogenic living tumor tissue *or* with cell membrane preparations obtained from neoplastic cells conferred an appreciable degree of heightened resistance against subsequent challenge with tumor isografts (figure 11, table XII). (The rationale for studying

TABLE XII

DEVELOPMENT OF ISOGRAFTS OF A STRAIN A SPONTANEOUS MAMMARY CARCINOMA IN MICE IMMUNIZED WITH MEMBRANE RICH FRACTIONS OF TUMOR CELLS AND OF LIVER AND KIDNEY CELLS

Young adult strain A mice were immunized by repeated subcutaneous injections of tumor cell or normal cell membrane preparations, the first injection being administered in Freund's complete adjuvant. Ten days after the last injection, the animals were challenged in each inguinal fat pad with living cells of the same tumor. The nature of the membrane preparation and other experimental details are presented elsewhere [102]. Two inocula of tumor cells (5×10^3) were given each animal 10 days after last immunization; animals were observed 90 days. There were three to six injections of tumor membrane fraction totaling 600 to 900 μg.

Pretreatment	Number of Tumors/Total	
Tumor membranes + Freund's adjuvant	2/70	(3%)
Normal membranes + Freund's adjuvant	22/46	(48%)

the immunogenicity of cell membrane preparations stemmed from the observations of many investigators that the neoplastic transformation is often accompanied by marked changes at the cell surface [130], [131], [132]; the expression of tumor associated antigens in cell membrane components is therefore a reasonable expectation. Moreover, only antigenic changes at the cell surface are likely to be of significance in the development of immunological resistance against antigenically foreign cells.)

(2) Immunization with *both* living tumor tissue and cell membrane preparations elicited the opposite effect, enhanced growth of the challenge tumor

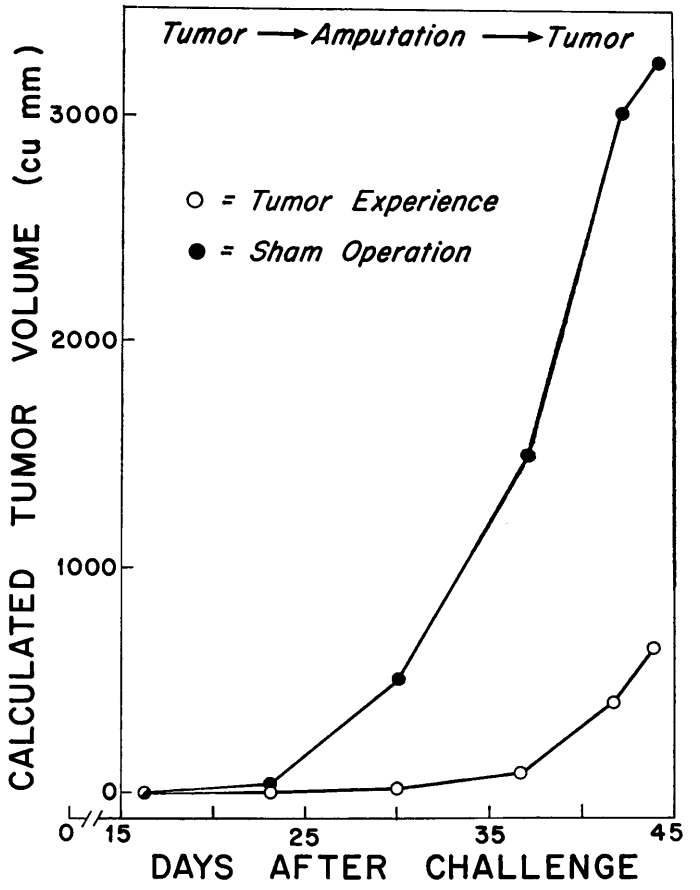


FIGURE 11

Development of isografts of a strain A spontaneous mammary carcinoma in mice immunized with living tumor tissue.

Young adult strain A mice received implants of a spontaneous mammary tumor in the right hind legs. When the tumors had developed to a size of approximately 50 to 100 cubic mm, the tumor bearing limbs were amputated. Six weeks later the mice were challenged with 5×10^3 living cells of the same tumor, injected into each inguinal fat pad. Control animals subjected to the same surgical procedures but receiving no immunizing tumor tissue, were similarly challenged. The figure shows the means of the calculated tumor volumes (10 mice per group) at several time intervals after tumor challenge.

isografts (figure 12). Enhanced tumor growth could also be induced passively by means of serum from immunized animals (figure 13), and is therefore tentatively assumed to represent the phenomenon of classical immunological

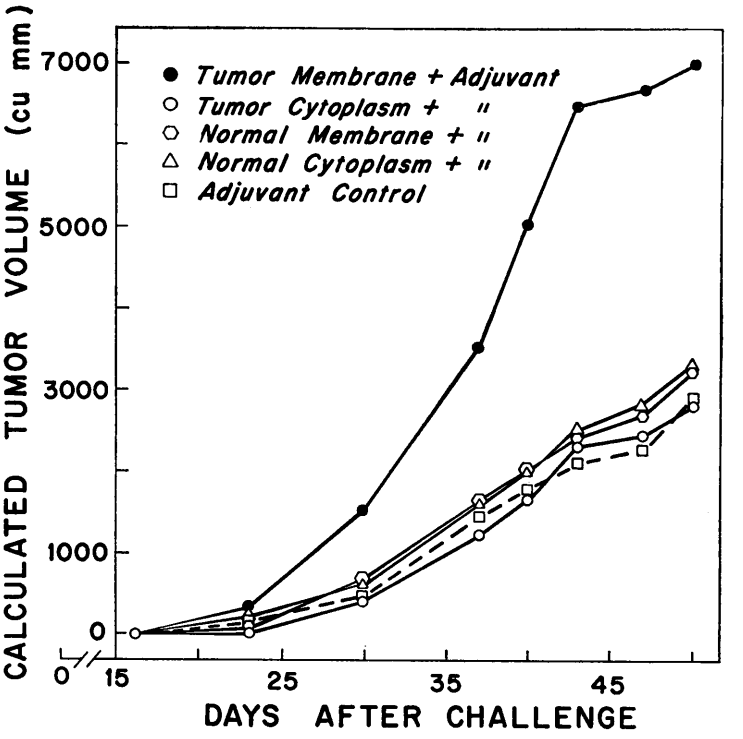


FIGURE 12

Enhanced growth of isografts of a strain A spontaneous mammary carcinoma in mice immunized with a living tumor graft *and* with a tumor cell membrane preparation.

(Data from [102].)

Young adult strain A mice received implants of a spontaneous mammary tumor or of an entire normal mammary gland in the right hind legs. When the tumors had developed to a size of about 200 cubic mm, the limbs bearing the tumors and also those carrying the normal mammary tissue were amputated. Three to four weeks later, the animals received single intraperitoneal injections of preparations of either membranous or cytoplasmic fractions derived from tumor cells or from a mixture of normal mammary, liver, and kidney cells; mice which had received implants of living tumor tissue were injected with the tumor cell preparations, and those which had been given living mammary glands were injected with the normal cell preparations. Each animal received 0.1 mg, dry weight, of the tissue preparations, suspended in complete Freund's adjuvant. A control group received adjuvant only. Ten to thirteen days after injection all animals were challenged with tumor implants placed into each inguinal mammary fat pad. The figure shows the means of the calculated tumor volumes (6 to 10 mice per group) at several time intervals after challenge.

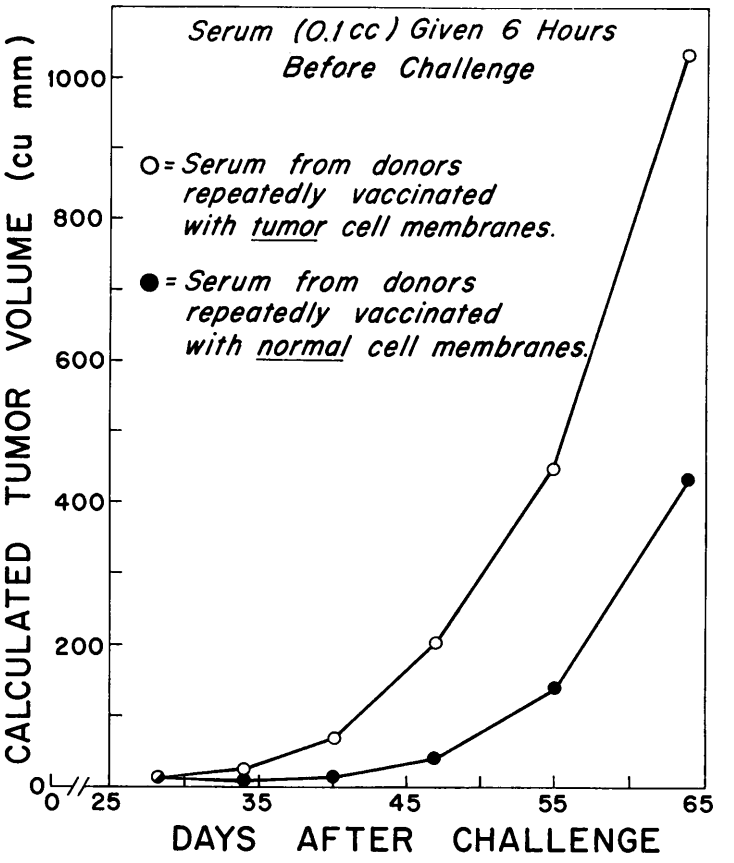


FIGURE 13

Passive induction of enhanced growth of isografts of a strain A spontaneous mammary carcinoma by means of serum from actively immunized donors. (Data from [102].)

The serum was taken from isogenic donors which had been immunized by repeated injections of a tumor cell membrane preparation, and which were found, upon tumor challenge, to have acquired a degree of heightened *resistance* (see table XII). The figure shows the means of the calculated tumor volumes (14 or 15 mice per group) at several time intervals after challenge with 5×10^4 viable tumor cells injected subcutaneously in each inguinal area.

It is thus evident that animals which display specifically acquired heightened resistance against a tumor may have in their serum factors (presumably, antibodies) which can confer the opposite effect, enhancement. This observation provides further indication that the nature of the manifestation of an immune response against foreign tissue may depend on the equilibrium of cellular and humoral immune factors [102].

enhancement [91], [92], [133] to [137]. Possible reasons for the opposite effects produced by the different immunization schedules are discussed in a detailed report based on these experiments [102]; a fully satisfactory explanation is still lacking, however.

(3) Enhancement induced with one tumor was manifested against other tumors as well (table XIII), suggesting a degree of immunological cross reactivity.

TABLE XIII

ENHANCED GROWTH OF ISOGRAFTS OF STRAIN A SPONTANEOUS MAMMARY CARCINOMAS IN MICE IMMUNIZED WITH LIVING TUMOR TISSUE AND WITH TUMOR CELL MEMBRANE PREPARATIONS DERIVED FROM THE CORRESPONDING AND FROM OTHER TUMORS
(Data from [102].)

The experimental details are similar to those described in figure 12. The animals were given an implant of living tissue followed by removal, and injection of corresponding cell membranes in adjuvant. There were ten animals per group.

Tumor Used for		Average Tumor Size at Termination (cm^3)
Immunization	Challenge	
A 2	A 2 (slow)	1.7 ± 0.5
A 3	A 2	0.5 ± 0.3
A 4	A 2	0.7 ± 0.4
Normal tissue	A 2	—
Adjuvant alone	A 2	0.3 ± 0.2
A 2	A 3 (slow)	2.3 ± 0.3
A 3	A 3	1.6 ± 0.3
A 4	A 3	1.7 ± 0.4
Normal tissue	A 3	1.3 ± 0.2
Adjuvant alone	A 3	1.4 ± 0.3
A 2	A 4 (fast)	2.5 ± 0.3
A 3	A 4	2.5 ± 0.3
A 4	A 4	2.4 ± 0.3
Normal tissue	A 4	1.3 ± 0.2
Adjuvant alone	A 4	1.5 ± 0.2

(4) Immunization with cytoplasmic fractions of tumor cells, or with any normal cell preparation, living or inactivated, failed to elicit either heightened resistance or heightened susceptibility to later tumor challenge.

It is apparent from these findings that infection of the test animals with MTV at birth does not always preclude the development of immunological reactions against MTV infected tumors. It would be tempting to draw the conclusion therefrom that the heightened tumor reactivity specifically acquired by such animals reflects the presence of a category of antigen(s) independent of the MTV. The failure to demonstrate heightened tumor reactivity in isogenic C3H mice under similar conditions could then, conceivably, be ascribed to their generally lower immunological abilities; it is also recognized that not every species and strain of animals responds equally to the same antigens. The better response of

the autochthonous and original C3H and C3H/2 tumor hosts could perhaps be explained as resulting from a greater antigenic stimulation in the course of the more prolonged, and biologically perhaps quite different, interaction between a tumor and its *primary* host. The heightened tumor resistance elicited in MTV infected mice by pretreatment with MER also supports the existence of an MTV independent antigenicity.

However, another interpretation of the findings in the strain A mice must also be entertained. The immunogenicity of mammary tumors in this strain *could* be dependent on MTV associated antigens, and the immunological reactivity of the animals despite MTV infection could be accounted for by an incomplete immunological tolerance (that is, unresponsiveness) to these antigens. It is well known that immunological reactions can occur against some "self" antigens under certain conditions, notably when the barriers between tissues carrying such antigens and the immunologically reactive tissues of the body are broken down as a result of accident, another pathological condition, or the agency of the experimenter. Although infectious MTV can be isolated from many organs, the characteristic viral B particles commonly thought to represent the MTV are expressed in most mice *only* in mammary tissue [138]. (Exceptions to this generalization have been reported, however, [144], [145].) The mammary gland of mice has recently been found to be an immunologically "privileged site," that is, transplants of foreign tissue will not be rejected, or be rejected later when implanted into the mammary gland than when placed into another area of the host [139]. It is perhaps possible, therefore, that MTV associated antigens, or at least those associated with the maturation of the formed B particle, may not be in effective contact with the immunological apparatus of the normal organism. They would accordingly not be treated as "self," and hence could evoke an immune response when brought into systemic contact with the animal. The absence of such a response in the C3H strain could still be viewed as the result of a general or a particular immunological deficiency, or it could arise from differences between these and the strain A mice in the degree of communication between mammary parenchyma and lymphoid tissue centers. Similarly, differences in such communication could be envisaged between implanted and autochthonous tumors.

Another mechanism of immunological responsiveness to MTV associated antigens in animals neonatally infected with MTV can be entertained. It has been shown by a number of investigators that a specific immunological unresponsiveness can be "broken" by immunizing the unresponsive animal with an antigen related to the one to which the tolerance is manifested [140] to [143], and it has been suggested that advantage could perhaps be taken of this fact to break specific immunological unresponsiveness against cancer associated antigens in man [146]. In the process of preparing MTV infected mammary tissues for immunization, and perhaps also as a consequence of the chemical changes which undoubtedly take place in neoplastic tissue in the course of progressive tumor growth, alterations of antigenic structure could well occur, with the result that

the immunized animals regain the ability to respond immunologically to the *unaltered* MTV associated antigens in the tumor challenge tissue. (It was thought until recently that a tolerance inducing period precedes that of immunological responsiveness in the ontogeny of animals. Recent studies suggest that this may not be true, however, and that ability to respond immunologically to an antigen may be requisite for the induction of specific tolerance against it [147], [148]. It is for this reason that the word "regain" is here employed.) On the other hand, evidence has recently been brought forward which suggests that a state of specific immunological tolerance cannot be broken by immunization with related antigen in the presence of large quantities of the "tolerogen" (that is, the tolerance inducing antigen). Although a full abrogation of tolerance (that is, production of antibodies with the same specificities as before induction of the tolerance) may not be attainable in the face of excess tolerogen, there could perhaps be elicited antibodies of different specificities, but still reactant with at least some of the antigenic determinants of the initial antigen [143].

The cross reactivity of the strain A tumors, as indicated by their ability to induce cross enhancement, is compatible with the postulation of an MTV associated antigenicity. However, the cross reactivity could also arise from the presence of antigen unrelated to the MTV but expressed in similar form in all, or most, tumors of this type.

7.6. *Discussion.* It is apparent from the aforegoing paragraphs that the nature of the antigen, or antigens, responsible for the immunogenicity of spontaneous mammary tumors and preneoplastic nodules remains to be determined. There is already available persuasive evidence that one or more of these antigens are associated with the MTV. How the MTV acts in the expression of this antigen(s) is still unknown. The information derived from experiments with MTV infected autochthonous and NIV infected "original" hosts, and from the more extensive studies in MTV infected isogenic strain A mice, are suggestive of the existence of another, virus independent antigen(s), but alternative explanations are also tenable at this point.

Considerable effort is currently directed in our laboratory to an elucidation of the biological nature of the tumor associated antigen(s). One study is directed at a determination of the immunogenicity of concentrated preparations of MTV purified by centrifugation in ficoll and/or rubidium chloride gradients, and then inactivated by physical and chemical means. In another experiment, C3H and A females are given injections of MTV infected normal or neoplastic mammary tissues, beginning several days before birth (that is, the injections are given the animals while they are still *in utero*) and maintained periodically thereafter, in an attempt to induce a high degree of specific immunological unresponsiveness. The animals are then immunized with living and inactivated tumor tissue, and their ability to develop immunity against isografts of the corresponding tumors and of other mammary tumors is studied. A third line of experimentation consists of immunizing BALB/c animals with isogenic mammary tumors of different viral status (MTV+, NIV+; MTV+, NIV−; NIV+, MTV−; and, MTV−,

NIV−) and challenging them with tumors of the same and of different viral background.

The practical implications of these studies are obvious. Effective immunological intervention in neoplastic diseases of man has been a much cherished, and an entirely elusive, dream of cancer investigators. If this dream appears less chimerical today than it has in the past, it is partly because of the accumulated observations in the area of experimental tumor immunology during the last decade, and partly because of the considerable progress in the general knowledge of immunological reactions during recent years. It is certainly true that major advances in human medicine are not always based on sound scientific background; indeed, some of the most important gains in the clinic owe more to a concatenation of luck, intuition, and chance occurrence than to a rational scientific approach. But efforts to interfere therapeutically, or prophylactically, with neoplasia have so far not been distinguished by such good fortune, and the laboratory investigator still has an unchallenged mandate here. And it seems to me that two questions which now urgently demand an answer before new approaches to immunotherapy and immunoprophylaxis of cancer become possible are: What is the biological nature of the antigen(s) specific to, or associated with, the neoplastic condition? And, what is the nature of the immunological deficiency which permits the progressive growth and colonization of antigenic neoplastic variants?

8. Studies on the nature of the specifically heightened reactivity against spontaneous mammary tumors

In preceding paragraphs, experiments have been described which show that heightened tumor resistance can be transferred by means of lymphoid cells from immunized donors, and heightened tumor susceptibility (presumably, immunological enhancement) by means of immune serum. These observations, as well as other findings already discussed, provide strong indication that the heightened tumor reactivities are of classical immunological nature.

Experiments are now in progress to study the interrelationship of humoral and cellular factors in heightened tumor reactivity, and the kinetics of the resistance and enhancement phenomena. It is beyond the scope of this paper to discuss these studies in detail, and, at any rate, much of the information is still incomplete. One finding will be singled out for brief mention, however, because of its intrinsic interest, and also because it has provided a much needed short term (though only semiquantitative), *in vitro* assay for the MTV.

In a series of investigations carried out by Dr. Phyllis B. Blair in our laboratory, it was found that rabbits and guinea pigs react with a specific immunological response to the MTV [149], [150]. This response could be demonstrated *in vitro* by reacting the immune sera with tissue preparations rich in MTV [151] in a dilute agar immunodiffusion system [152]. A precipitation line specific for the interaction of MTV—the virus is small enough to just move in a dilute

agar gel—and the sera of MTV immunized animals was detected [151], [153]. The MTV specificity of this line was shown by the complete correlation between its occurrence and the presence of MTV in the immunizing as well as in the test tissues [153]; and, electron microscopic examination of the specific precipitation line revealed the presence of clumps of B particles, surrounded by a matrix of granules suggestive of globulin (antibody?) molecules [154]. (Comparison of the MTV with the NIV of C3Hf animals revealed a degree of cross reactivity [155], not unexpected in view of the morphological similarities and common nodule inducing properties of the two agents; however, there is reason to believe that the NIV does not share all of the antigenic characteristics of MTV (Blair and Weiss, unpublished data).)

The development of a specific immunodiffusion assay for the MTV now permits its detection in a matter of weeks (if the unknown tissue is used for immunization) or days (if the unknown tissue is used as reactant in the plates or for absorption of a known antiserum), a considerable improvement over the most rapid assay method available until now, the three to four months long noduligenic assay developed by Nandi [156]. However, the pertinence of these findings to the present discussion does not lie in the development of an *in vitro* assay for MTV, or in the facilitation of studies of the relationship between MTV, NIV, and possible other members of this family of oncogenic agents. It comes, rather, from the availability of a rapid, specific, and sensitive method for detecting small amounts of antibody directed against the MTV.

The immunodiffusion assay was, accordingly, applied to the sera of mice which had been immunized to evoke heightened tumor resistance. Contrary to the reports of earlier investigators that mice cannot produce antibody against MTV [157], [158], [159], it was found that a large proportion of mice exposed experimentally, or naturally, to the MTV produced the line-forming (that is, precipitating) antibodies [160].

It is not known as yet whether these antibodies play a role in heightened tumor resistance, or, for that matter, whether *any* humoral antibody is involved in resistance phenomena against these neoplasms. On the other hand, the antibodies may well be responsible for the induced tumor enhancement. If nothing else, the demonstration of immunological reactivity of mice against one component of neoplastic cells—the oncogenic virus—indicates that the immunological nature of the heightened reactivity against the intact neoplastic cells need not be considered a surprising finding.

9. Conclusion

Concluding statements more sweeping than those tentatively advanced throughout the text of this communication would not be justified. Should I be given the pleasure of presenting another paper at the next Symposium, five years hence, I might perhaps be able to write something in the nature of a true conclusion. For the time being, a realistic evaluation of the still very uncertain ground

treaded by investigators in the area of tumor immunology limits me to a summary recapitulation of the several more pertinent facts which seem to have emerged from our studies.

(1) Spontaneous mouse mammary carcinomas infected with MTV are highly immunogenic in isogenic animals free of the virus. They are sometimes also immunogenic in the MTV infected animals in which they first arose, though less so; and they are able to elicit a significant degree of heightened reactivity in isogenic mice of at least one strain despite the infection of the hosts with MTV. Tumors arising in mice not infected with MTV are also moderately immunogenic in the original hosts.

(2) The specifically acquired heightened reactivity against the mammary tumors is usually manifested by increased resistance, but under certain circumstances by increased susceptibility. The immunization procedure is an important determinant of which of these reactions is evoked.

(3) Heightened tumor reactivity can be induced by immunization with living tumor tissue and with preparations of tumor cell membranes.

(4) Specifically acquired heightened tumor resistance can be transferred to normal isogenic mice by means of immune lymphoid cells, and the heightened susceptibility can be induced passively by means of immune serum. This, and other observations, indicate that both these consequences of a previous experience with neoplastic tissue are immunological in nature.

(5) At least one antigen responsible for the immunogenicity of MTV infected mammary tumors is already expressed in infected preneoplastic and normal mammary tissue. There is considerable cross reactivity among different MTV infected mammary tumors and preneoplastic tissues, apparently the result of the similar or identical nature of this antigen in all MTV infected mammary tissue. It appears not unlikely that there exists in addition another antigen, or category of antigens, not associated with the presence or activities of the MTV.

(6) Mice are able to make antibodies which react specifically with the MTV.

(7) Treatment of mice with a tubercle bacillus fraction known to heighten immunological reactivity to unrelated antigens increases significantly their reactivity against tumor isografts and autochthonous tumors, and against the development of the preneoplastic alveolar nodules.

The experimental work here described is based on collaborative studies carried out in our laboratory during the past five years with Drs. M. A. Attia, P. B. Blair, K. B. DeOme, and D. H. Lavrin, Mrs. R. S. Bonhag, Mrs. D. Burton, Mr. M. Dezfulian, Miss C. Steinkuller, and Mr. J. Vaage. The technical assistance of Mrs. P. Leslie is gratefully acknowledged.

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