Demonstration by Gel Diffusion of Antigen in Spontaneous Mouse Tumors

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Summary

Spontaneous mammary tumors from random-bred Swiss Webster mice were pooled and used, with Freund's adjuvant, to immunize rabbits. The rabbit antiserum was absorbed with normal mouse tissues and tested by gel immunodiffusion with a 2nd group of tumors. The latter were pooled to form 3 distinct groups—strong, weak, and negative reactors. The 3 tumor pools were then used to immunize 3 additional groups of rabbits. The rabbit antisera harvested from the secondary groups were absorbed with spleen homogenate, serum, and mammary gland homogenate from normal mice of the same origin. If antiserum reacted positively in gel diffusion with tumor homogenate, it was also absorbed with contralateral mammary gland extract from the same tumor mice. After the final absorption, the rabbit antisera were tested in gel immunodiffusion against tumor homogenate, serum and extracts of 2 organs from the same tumor-bearing mice, serum and 4 organ extracts from normal mice, extracts of normal mouse embryo, extracts of 2 transplantable mammary carcinomas, and extracts of contaminating bacteria. Of 15 rabbits immunized, 12 produced antisera which reacted with the tumor extract, but not with the other materials. Each rabbit produced the reacting antibodies for a short period of time only. Extracts of very small tumors did not react with the rabbit antisera.

The results suggest that there are distinctive antigens in spontaneous tumors, or that the concentration of some normal antigens may be greatly increased. The antigens in question are not weak, but may require special technics for identification.

Introduction

The importance of an immunologic approach to cancer therapy depends on the existence of antigenic differences between normal and malignant tissue. Several investigators (9, 16, 18, 20, 22, 23, 25, 28, 30, 31) have presented evidence which suggests that in malignant tumors there are antigens which are not ordinarily found in normal tissues. Many of these studies were done on chemically induced or on transplanted tumors, but some were done on human malignant tumors (1-8, 11, 14, 15, 19, 27, 32). Most studies on spontaneous mouse tumors which suggested the presence of distinctive antigens have utilized indirect methods of demonstrating the antigens. Accordingly, it seemed important to use more direct methods in examining spontaneous mouse tumors for tumor antigens.

Materials and Methods

EXPERIMENTAL ANIMALS. Random-bred female Swiss Webster mice bearing spontaneous mammary carcinomas were obtained from 6 separate animal breeders—2 in New York, and 1 each in Pennsylvania, Missouri, Delaware, and Indiana. The mice were approximately 8-10 months old and in good general health. They were housed and handled so as to avoid any contact between animals from 2 separate sources.

Matched animals without tumors from the same breeding farms were used as sources of normal mouse tissues and serum. Separate hair clippers were used for mice from each farm. Mice were kept for 1-2 weeks after arrival, and if any evidence of infection was seen, were eliminated.

All mice were sacrificed by cervical dislocation or exsanguination. The skin was treated with 75% by volume ethyl alcohol which was not denatured, and the alcohol allowed to dry before the skin was incised. Tumors and organs were removed, using aseptic technics, including sterile gloves, autoclaved instruments, and autoclaved operating platforms. Tumors were examined carefully, and any which showed evidence of necrosis or infection were discarded.

TISSUES USED FOR TESTING. Tumors and tissues which were removed were frozen and stored at -20°C. The tissues removed and frozen for testing as antigens against the rabbit antiserum were:

1. Spontaneous mammary carcinoma from Swiss Webster mice.
2. Mammary tissue from the unaffected side of the same Swiss Webster mice bearing spontaneous tumors.
3. Spleen from the same mice with spontaneous tumors.
4. Liver from the same mice with spontaneous tumors.
5. Serum from the same mice with spontaneous tumors.
6. Mammary glands from nonpregnant, nonlactating, normal mature female Swiss Webster mice.
7. Mammary glands from pregnant, nonlactating, normal mature female Swiss Webster mice.
8. Mammary glands from nonpregnant, lactating normal mature female Swiss Webster mice.

Received for publication July 23, 1965; revised January 7, 1966.
ANTIBODY RESPONSE. The antisera harvested from the rabbits demonstrated a transient antibody response which depended on the time of harvesting and which persisted for a period not exceeding 10 days. In many cases, only the antisera from a single bleeding was positive. Of the 15 rabbits tested, the sera of 12
showed a positive reaction to a tumor pool, even after absorption with contralateral mammary tissue from the same tumor-bearing mice. The time pattern of response differed with each rabbit, so that no conclusions could be drawn about the best time to harvest antiserum. Therefore, in describing below the animals whose sera reacted with the tumor antigen, we mean that the serum from at least 1 bleeding reacted.

The unabsorbed sera of all rabbits reacted with both tumor extract and extract of normal organs. However, after the absorption procedure, none of the sera reacted with extract of normal organs, and 12 of 15 still reacted with the tumor extract. After those 12 serum samples were absorbed with extract of mammary tumor, the reactivity was no longer present.

Figs. 1 and 2 illustrate the observation that the rabbit antiserum absorbed with homogenates of serum and normal organs did not produce precipitin lines with such homogenates but did produce precipitin lines with tumor homogenate.

Individual tumor homogenates. Fig. 3 demonstrates the reaction of 6 individual tumor homogenates with rabbit antiserum previously absorbed with normal mouse mammary glands. There were distinct lines between each of these tumor homogenates and the antiserum. The pattern suggests that each of the 6 tumors may contain an antigen which is the same, or very nearly the same, as those in the other tumors.

Comparison between tumor and mammary gland. Fig. 4 demonstrates the reaction or lack of reaction of the absorbed rabbit antiserum with the original tumor pool, a secondary tumor pool of positive reactors, and a pool of normal mouse mammary glands. There were distinct lines between the antiserum and both tumor pool homogenates, but not between antiserum and normal mouse mammary gland homogenate.

Secondary response. A secondary response to the tumor extract could not be demonstrated in rabbit antiserum harvested after the 3rd inoculation of tumor extract and absorbed with the normal mouse materials.

Relation of tumor size to antigenicity. The reactivity of the antiserum of rabbits inoculated with the 2nd group of tumors is shown in Table 1. The occurrence of precipitation between individual tumor homogenates and a known positive antiserum was as follows: negative reactions, 12; strong positive reactions, 9; weak positive reactions, 6. The consulting pathologist found no histopathologic difference between the negatively and positively reacting tumors submitted to him. No consistent pattern was found which might relate the type of reaction to the breeding farm origin. However, comparison to tumor size index suggested a possible relationship of reactivity to tumor size, and this comparison is shown in Chart 1. Only 25 of the 27 tumors are represented in this chart since tumor size was not measured in 2 animals.

The size indices of the E0771 tumors in the C57BL/6 mice ranged from 170 to 320, with a mean of 260. Thus, they were significantly larger than the spontaneous tumors used, including those which gave a strongly positive response. The E0771 tumor extracts reacted negatively with the rabbit antiserum.

Reactions with other extracts. The absorbed rabbit antiserum which gave positive reactions with homogenates of pooled spontaneous tumors gave negative reactions with the other 12 extracts of mouse tissue listed above. In addition, the supernatant of the bacterial broth culture made with contaminating bacteria did not react with any of the absorbed antiserum, and the bacteria centrifuged from the broth did not in further absorption procedures remove the antibody which reacted with mammary tumor extract.

Discussion

The decision to study spontaneous tumors in random-bred mice was made after careful consideration of many relevant factors. Random-bred mice have many disadvantages for cancer research, compared to inbred mice. One of the major disadvantages, of course, is the genetic and antigenic disparity between different random-bred mice. Nevertheless, through appropriate experimental design in which tissues from each mouse are used as additional controls, this disadvantage is neutralized. Furthermore, inbred mice may have the same disadvantage, but in a less recognizable form. Although inbred mice are closer to one another genetically and antigenically than are random-bred animals, the inbred mice are by no means identical antigenically. Despite the many disadvantages and inconveniences in working with spontaneous tumors in random-bred mice, there is 1 major advantage. Findings in random-bred animals with spontaneous tumors are more likely to be relevant to the human problem, in which spon-

<table>
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<th>No. of rabbits inoculated</th>
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<th>3</th>
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<td>No. of rabbits whose antisera gave positive reaction to tumor pool</td>
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<td>0</td>
<td>6</td>
<td>3</td>
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<td>0</td>
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<tr>
<td>No. of rabbits whose antisera gave positive reaction to pools of normal mouse materials</td>
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* Also tested against other pooled extracts.

† Note that this is after absorption of the rabbit antiserum with normal mouse materials.
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Chart 1. The relationship of tumor size to reactivity in gel diffusion.

Antigen in spontaneous tumors develop in random-bred individuals. By contrast, findings on transplantable tumors in inbred animals might be less relevant to the problem of human cancer. Hirsch (12, 13) has pointed out the need and importance of studies on spontaneous tumors.

In the past, reports of identification of tumor antigens have been criticized because of inadequate controls. For example, Witebsky (29) points out that confusion may result from the presence of organ, as well as blood, isoantigens. Accordingly, in this study, particular care was taken in utilizing controls. At this point, it is not possible to determine whether our results are based on a single discrete antigen, or on a group of closely related antigens. Accordingly, when the term “tumor antigen” is used in this discussion, it should be understood as referring also to a possible group of closely related antigens.

The antigen which has been demonstrated does not seem to be an organ or blood isoantigen because it was not absorbed by extract of mammary glands of normal mice, lactating and non-lactating, by extracts of contralateral mammary gland, or by extracts of organs and sera of the same tumor mice. The possibility of the antigen having a bacterial origin seems to be extremely unlikely for several reasons. The level of bacterial contamination of our preparations was quite low; it was never more than 100/ml. By contrast, the levels of bacterial contamination which Kamp-schmidt and Upchurch (17) found in some transplantable tumors and which they believed caused effects on the host average about 10^9/ml. In addition, the supernate of a broth culture of the contaminating bacteria did not react with the rabbit antiserum, and the cultured bacteria, at much higher concentrations, did not absorb the antibody which reacted with the tumor extract.

The antigen in question does not seem to be one which is common to immature, rapidly multiplying cells, since it was not found in extract of mouse embryo, or in extract of 2 other mouse carcinomas.

In view of the observation that the antigen is somewhat more readily demonstrated in large tumors, the possibility was considered that it might be the result of hypoxia, necrosis, or some other degenerative change occurring in relatively large tumors. However, this possibility, though not completely ruled out, seems remote for several reasons. The centers of the tumors used did not show any evidence of necrosis or degeneration. Of course, this does not remove the possibility of early changes of a biochemical nature which might not be visible. However, a series of 20 large E0771 carcinomas was also tested. These transplantable tumors were deliberately allowed to grow to a rather large size, so that their average size index, 260, was greater than the size of the spontaneous tumors which gave a positive response. Therefore, if the tumor antigen were actually an artifact caused by early degenerative changes in the interior of a large tumor, one would expect a high proportion of the large E0771 carcinomas to show the same artifact. However, none of the 20 transplantable tumors showed such an antigen. This suggests that the tumor antigen is not an artifact but is closely associated with the spontaneous tumors.

A crucial question is whether the observed antigen is an abnormal one, found only in spontaneous mammary tumors, or whether it is an antigen, found in small amounts in normal tissues, but present in much larger amounts in the tumors. This question cannot be answered by the experiments described. Indeed, it is difficult to see how an inability to find a particular antigen in normal organs can ever be definite proof of the complete absence of that antigen, since there is always some threshold of sensitivity for all tests. A negative finding means that the amount of material which is present is less than the threshold of the particular test used.

In the case of the tumor antigen, it is quite possible that we are dealing with a “normal” antigen present in grossly abnormal amounts. On the basis of our experience with the appearance of the precipitin lines and some concentration experiments, it can be estimated that if the tumor antigen is present in normal mouse
directed against the antigen has a deleterious effect on the cells. However, even if the latter proves to be the case, the finding pointed out above, a virus, or an excretory product of the tumor, might still be of considerable significance, since, as pointed out by Rapport and Graf (24) antigens can be exploited if the antibody to the tumor antigen also seems significant. We found that out of 15 rabbits inoculated with tumor pools, 12 responded at some time with demonstrable antibody levels. However, such levels never were present for over 10 days and most were obtained only on a single bleeding. If we had pooled the antiserum samples from each rabbit the level of antibody would have been between 1/2 and 1/4 as great, and there is a distinct possibility that such pooled rabbit antiserum samples would not have produced reactions with the tumor antigen.

The apparent lack of a demonstrable secondary response to the tumor antigen may have little or no significance. The normal mouse proteins are apparently more antigenic to rabbits than is the tumor antigen, and the secondary response could have been concentrated on the normal mouse proteins. In addition, the timing which was selected for the booster inoculation may have been unfavorable. Perhaps an earlier or later booster inoculation might have resulted in a definite secondary response to the tumor antigen. On the other hand, there also exists the possibility that the lack of a secondary response to the tumor antigen has a significance which is not yet apparent.

Sinkovics et al. (26) have stated, "The antigenicity of spontaneous tumors, on the other hand, is relatively poor." Apparently some other investigators have not been successful in demonstrating antigens in spontaneous mouse tumors. However, the results in the present study suggest that the antigenicity of spontaneous tumors may not be poor at all but may require special technics for demonstration. In our studies, the antiserum was absorbed 3 times before reaction with the tumor extract. Each absorption involved a dilution of the antiserum to 40% of its original concentration. Accordingly, the final concentration of antibody was only 6.4% of the original concentration in the antiserum, and of course, it was diluted still further in the gel diffusion process. Nevertheless, it produced distinct precipitin lines with antigen. This suggests that the antigen in question is a rather strong, rather than a weak, antigen since it produced a strong antiserum.

The difficulty which has arisen in the past in attempts to demonstrate antigens in spontaneous mouse tumors may stem from 2 roots. First, the tumors studied may not have been large enough, and all the available antigen may have been bound to host antibodies. Secondly, because the rabbit's antibody response to the mouse tumor antigen is so short-lived, several successive bleedings of the rabbits would be needed to obtain a serum sample with a demonstrable antibody level. Pooling of the rabbit antiserum samples would reduce the chances of detecting the antigen-antibody reaction.

Acknowledgment

We are grateful to Dr. Carlos Perez-Mesa, Pathologist at the Ellis Fischel Cancer Hospital, for his kindness in reviewing the slides of the tumors.

References

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Fig. 1. Gel immunodiffusion of absorbed antiseraum with normal and malignant mouse tissue extracts. Well No. 1, absorbed rabbit antitumor serum. Well No. 2, pooled mouse mammary tumor homogenate. Well No. 3, pooled normal mouse mammary gland homogenate. Well No. 4, pooled normal mouse spleen homogenate. Well No. 5, pooled normal mouse serum.

Fig. 2. Gel immunodiffusion with tissue pools from tumor-bearing mice. Well No. 1, absorbed rabbit antitumor serum. Well No. 2, pooled mouse mammary tumor homogenate. Well No. 3, pooled spleen homogenate from tumor mice. Well No. 4, pooled serum from tumor mice. Well No. 5, pooled contralateral mammary gland homogenate from tumor mice. Well No. 6, pooled liver homogenate from tumor mice. Well No. 7, pooled mammary gland homogenate from normal mice.

Fig. 3. Gel immunodiffusion reactions of individual tumors with absorbed antiseraum. Well No. 1, absorbed rabbit antitumor serum. Wells No. 2-7, individual spontaneous mouse mammary tumor homogenates.

Fig. 4. Gel immunodiffusion reactions between absorbed rabbit antiseraum, original tumor pool, and 2nd, positive tumor pool, compared to normal mouse mammary glands. Well No. 1, absorbed rabbit antitumor serum. Wells No. 2 and 5, Original mammary tumor homogenate pool. Wells No. 3 and 6, secondary mammary tumor homogenate pool of positive reactors. Wells No. 4 and 7, pooled normal mouse mammary gland homogenate.
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