Antibodies to Spontaneous and Methylcholanthrene-induced Tumors in Inbred Mice

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Summary

Isoimmunity to a methylcholanthrene-induced fibrosarcoma and to a spontaneous breast adenocarcinoma was induced in female C3H/HeN mice. Circulating antitumor antibodies specific for the particular immunizing tumor were detected in the immune mice. These antibodies did not appear until the primary tumors were surgically removed. Measurable amounts of antibody could be detected as long as 3.5 months following immunization. Specific adherence of these antibodies to tumor tissue was demonstrated with fluorescent antibody techniques. The presence of these antibodies correlated with the manifestation of increased host resistance to subsequent isologous tumor transplants.

Introduction

In a previous study (23), inbred C3H/HeN mice were shown to develop a specific immunity to a spontaneous adenocarcinoma of the breast as well as to a methylcholanthrene-induced fibrosarcoma when these tumors were allowed to grow temporarily in the hind limb. The degree of immunity observed was dependent upon the time interval between removal of the immunizing tumor and inoculation of a 2nd tumor transplant, and no immunity developed if the 1st tumor was not removed. In this paper evidence will be presented for the existence of circulating antitumor antibodies in immunized mice, which are not detectable in mice in which tumors are allowed to remain in situ. Higher levels of antibody were observed in animals which exhibited greater degrees of immunity.

Materials and Methods

Preparation of Tumor Cell Suspensions

Tumor-bearing animals were sacrificed by decapitation and the tumor tissue was aseptically excised and trimmed of necrotic and fibrous tissue. The tumor tissue, suspended in Hanks' balanced salt solution without antibiotics was then passed through a Snell cytosieve (25). Tumor cell viability was determined by the dye exclusion technic, with 0.5% trypan blue, and cells were counted in a hemocytometer.

Immunization

Female C3H/HeN mice (6 weeks old) were inoculated in the hind limb with single cells suspensions from either a spontaneously arising adenocarcinoma of the breast or the second transplant generation of a methylcholanthrene-induced fibrosarcoma. Tumors grew in all mice, and when the tumors were 1 cm in diameter the tumor-bearing limbs were amputated by direct cautery. Non-tumor-bearing mice of the same strain were subjected to hind limb amputation at the same time and served as controls. An additional control group consisted of tumor-bearing mice in which the tumors were left in situ and no amputations were performed.

Antibody Titrations

Mice were bled from the ophthalmic venous plexus into heparinized capillary pipets. Blood from mice in each experimental group was pooled and centrifuged at 1500 x g. The plasma was then removed and stored at -87°C. Antibody titers were determined by a modification of the tanned red cell hemagglutination method of Boyden (5).

Sheep erythrocytes stored in Alsever's solution at 4°C were washed 3 times in phosphate-buffered saline, pH 7.2 (PBS 7.2). A 2.5% suspension of the washed cells in 1:20,000 tannic acid (Mallinckrodt reagent grade) in PBS 7.2 was incubated in a 37°C water bath for 30 min and then washed once with PBS 7.2 and resuspended to a 2.5% suspension in normal saline. A portion of these cells was then washed once with 1% normal rabbit serum in saline (1% NRS) and a 2.5% suspension was made in 1% NRS for use in control tubes. The normal rabbit serum had previously been absorbed with thrice-washed sheep erythrocytes for 1 hr at room temperature.

The remainder of the tanned red cells were sensitized as follows: To 1 volume of a 2.5% suspension of tanned red cells in normal saline were added 4 volumes of the antigen preparation in phosphate-buffered saline, pH 6.4 (PBS 6.4). The suspension was incubated at room temperature for 15 minutes and then centrifuged at 800 x g for 15 min. The sedimented red cells were washed once with 1% NRS and resuspended to a 2.5% concentration in 1% NRS.

The antigen was prepared by a modification of the technic described by Aizawa and Southam (1). Tumor tissue was homogenized in 5 volumes of PBS 7.2 for 10 min at high speed in a Virtis 45 homogenizer. The homogenate was centrifuged at 3000 x g for 15 min, and the supernatant was saved. To the sediment were then added 3 volumes of PBS 7.2 and this mixture was homogenized for 10 min at high speed.
utation at 3000 \times g for 15 min, the sediment was discarded and the supernatant combined with that obtained from the first homogenate. The combined supernatants were then centrifuged at 7000 \times g for 30 min. The sediment was discarded, and this supernatant constituted the antigen preparation. All procedures were performed at 0°C. The protein concentration of the antigen preparations was determined by the method of Lowry et al. (17).

For each titration the mouse plasma was absorbed with thrice-washed sheep erythrocytes for 1 hr at room temperature and diluted serially in 1% NRS, starting at a dilution of 1:2.5. To 0.5 ml of each serum dilution was then added 0.05 ml of the 2.5% suspension of sensitized red cells in 1% NRS. In addition, 0.05 ml of tanned red cells which had not been incubated with antigen was added to 0.5 ml of each serum dilution to provide controls. Additional controls run in duplicate consisted of 0.05 ml of sensitized cells with 0.5 ml of 1% NRS, and 0.05 ml of tanned unsensitized cells with 0.5 ml of 1% NRS. The tubes were incubated at room temperature for 2 hr and then at 4°C overnight. The tubes were read for agglutination at the end of 2 hr and again the next morning.

Fluorescein-Labeled Antibody Studies

Mouse plasma was labeled with fluorescein by the method of Rinderknecht (24). Mouse plasma (2 ml) was added to 10 mg of fluorescein isothiocyanate on Celite 10% (Calbiochem, Los Angeles, Calif.), and the mixture was shaken for 3 min. The Celite reagent was then removed by centrifugation, and the plasma was passed through a Sephadex G-25 column to remove unbound fluorescein.

Frozen sections of tumor or isologous mouse liver were counterstained for 45 minutes with lissamine rhodamine bovine albumin (Microbiological Associates, Bethesda, Md.) and then washed 3 times with PBS 7.4. The fluorescein-labeled immune mouse plasma was absorbed with bovine liver powder and then applied to the tissue. After 45 min the sections were again washed 3 times in PBS 7.4. The sections were then mounted in 25% glycerine in PBS 7.4 and examined under ultraviolet illumination with a Schott UG 1 exciting filter and a Schott GG 9 barrier filter. Photomicrographs were obtained with AnSCOchrome 100 daylight type film.

Results

In order to determine the optimal concentration of the antigen preparation with which to sensitize the tanned sheep erythrocytes, rabbit antisera to these preparations were made. These antisera were absorbed with thrice-washed sheep red cells and with a saline homogenate of whole normal C3H/HeN mice for 1 hr at room temperature and overnight at 4°C. The sera were then titered against tanned sheep red cells sensitized with antigen dilutions, respectively, 0.125, 0.5, 3.125, 6.250, and 9.375 mg of protein/ml of tanned cells to be sensitized. The antigen concentration giving the highest titer (1:1280) was found to be 3.125 mg. This antigen concentration occasionally produced spontaneous agglutination in control tubes, but lower concentrations produced lower titers. Therefore, 2 antigen dilutions were selected, one containing 1.5 mg and the other 3.125 mg of protein/ml of tanned cells to be sensitized. All titrations on the mouse plasma were performed in duplicate using cells sensitized with each of these antigen concentrations.

Fifty-two mice were immunized against the methylcholanthrene-induced fibrosarcoma by allowing the tumor to grow in the hind limb for 8 days and then removing it by amputation. The mice were then bled 3 and 9 days after amputation. On both Days 3 and 9, titers of 1:20 (4 tubes) were obtained by using sheep red cells sensitized with the fibrosarcoma antigen preparation. When these plasmas were titrated with tanned red cells sensitized with the antigen extract prepared from the spontaneous carcinoma, no titers were observed. No antibodies could be demonstrated in the plasma of stock mice. Fifty-five non-tumor-bearing mice which had been amputated at the same time as the tumor-bearing group were also bled on Days 3 and 9 after amputation. When plasma from these mice was studied no titers were obtained on either day.

Two weeks after amputation, these two groups of mice were challenged with 1000 viable cells (viability was determined by the exclusion of trypan blue) from the 3rd transplant generation of the same fibrosarcoma. In addition, 31 stock mice were inoculated with the same tumor cell suspension to serve as additional controls. Only 4 of the 52 immunized mice developed tumors, whereas tumors arose in 51 of the 55 amputated control mice and in 29 of the 31 stock mice.

The 48 mice in the immunized group which did not develop tumors were bled at 35, 85, and 105 days postamputation. The plasma from Day 35 gave a titer of 1:10 (3 tubes) when tested against the fibrosarcoma antigen preparation, whereas the plasma from Day 85 gave a titer of 1:20 (4 tubes). By Day 105 the titer had decreased to 1:5 (2 tubes).

Fifty-five mice were immunized against the spontaneous breast adenocarcinoma. This tumor grew more slowly than did the fibrosarcoma, and amputation was performed 16 days after the initial tumor transplantation. At this time 55 non-tumor-bearing mice were amputated. Both groups of mice were bled 3 days after amputation. A titer of 1:10 (3 tubes) was demonstrated in the plasma of the immunized mice when the breast carcinoma antigen was used, but no reaction occurred when the fibrosarcoma antigen was used. No titer was detected in the plasma from the amputated control mice. These 2 groups of mice were challenged 7 days after amputation with 1000 viable cells from the 1st transplant generation of the same breast carcinoma. Thirty stock mice were inoculated at this time with 1000 tumor cells from the same cell suspension. In the immunized group, 33 of the 55 mice developed tumors, whereas tumors arose in 49 of the 55 amputated control mice and in 26 of the 30 stock mice. The 22 immunized mice in which tumors did not develop were bled on the 55th postamputation day. A titer of 1:10 (3 tubes) was still present.

A further study was undertaken in an attempt to detect naturally occurring antitumor antibodies in tumor-bearing mice in which the tumor was not removed. Accordingly, 90 mice were inoculated in hind limb with the fibrosarcoma. Two weeks later, when the tumor had reached a size of 1-2 cm, 30 animals were bled and the remaining 60 mice were each given an injection of 1000 viable tumor cells from the same transplant generation (3rd) of the fibrosarcoma into the contralateral hind limb. Eighteen stock mice received a similar injection of the same cell suspension to serve as controls. The mice were then housed individ-
usually to provide optimal conditions for survival. No circulating antibodies were demonstrated in the plasma of the 30 tumor-bearing mice, and the 2nd tumor transplants grew in 58 of the 60 animals. Tumors also appeared in 16 of the 18 controls.

Samples of the plasma obtained on the 3rd and 9th postamputation days from the mice immunized with the fibrosarcoma were labeled with fluorescein. Each of these immune plasma preparations was tested on frozen sections of the fibrosarcoma and normal isologous liver. When these sections were examined microscopically under ultraviolet illumination, bright fluorescence was observed on the tumor sections. This fluorescence was concentrated primarily in the cytoplasm of the tumor cells, and very little staining of the nuclei was noted. The connective tissue stroma was not stained. No appreciable fluorescence was seen on the sections of isologous mouse liver. Both plasma preparations reacted identically. When normal C3H/HeN plasma, labeled with fluorescein in a similar manner, was tested on frozen sections of the fibrosarcoma and normal isologous liver, no fluorescence was observed.

An attempt was made to determine whether or not the absence of circulating antibody in the unamputated tumor-bearing mice might be due to adsorption of antibody by the tumor, thereby removing it from the circulation. A frozen section of the fibrosarcoma which had grown in a mouse for 14 days was treated with fluorescein-labeled rabbit anti-mouse globulin (Microbiological Associates, Bethesda, Md.). No fluorescence was observed. If mouse immune globulin was adsorbed on the tumor, its concentration was too low to permit detection by this method.

**Discussion**

The existence of tumor-specific antigens in sarcomas induced in mice by methylcholanthrene and other carcinogenic hydrocarbons, and the production of isoimmunity to these chemically induced tumors, have been well established through the investigations of Foley (9), Klein et al. (16), Old et al. (20), and Prehn and Main (21, 22). Old et al. (20) attempted to detect tumor-specific antibody, both in vitro and in vivo, in hosts highly immunized against isogenic chemically induced tumors. Their results were essentially negative although the animals tested were highly resistant to transplants of isogenic tumor cells. However, the techniques in vitro did not include inadvertent rejection of tumor which could not have been detected.

Evidence that tumor-specific antigens capable of inducing isoimmunity also exist in the spontaneous breast carcinomas of mice has been slowly accumulating over recent years. In 1958 Hirsch et al. (14) reported an increase in the survival times of mice in which an implant of a spontaneously arising mammary adenocarcinoma had been excised and a 2nd, challenge implant immediately introduced. In 1962 Morton (19) reported that in isologous mice the temporary growth of transplants of a spontaneous mammary carcinoma conferred a significant degree of immunity to subsequent transplants of the same tumor. More recently, Weiss et al. (26, 27) have studied the immunity to spontaneous mammary carcinomas arising in female C3H/Crgl/2 mice that had received transplants of an outgrowth of a pre-cancerous hyperplastic alveolar nodule (HAN) derived from a C3H/f/Crgl female. These tumors were excised and subsequently reimplanted into their original hosts as well as into previously untreated isologous mice, and the tumor growth in the autochthonous hosts compared with the growth of the tumor in isologous recipients. In approximately half of the 25 tumors thus studied significant resistance to tumor growth was evidenced by the autochthonous animals. However, in a number of instances tumor growth in the original hosts appeared to have been enhanced. In similar experiments, 6 spontaneous mammary carcinomas arising in old multiporous breeders exhibited growth suppression when retransplanted into their autochthonous hosts. A recent report from this laboratory (23) has confirmed the presence of tumor antigens in another spontaneously arising mammary carcinoma, and has demonstrated the induction of significant immunity to this tumor in isologous mice. Although all the above authors succeeded in producing varying degrees of tumor isoimmunity, the presence of circulating antitumor antibodies in animals so immunized has not been demonstrated hereafter. It is not altogether surprising that tumor antigens, which are obviously quite active immunogenetically, should also elicit circulating antibodies. The single reported attempt by Old et al. (20) to detect such antibodies might have been successful had more sensitive technics (i.e., tagged red cell hemagglutination or complement fixation) been used to detect the presence of low titers of antibody. In addition, since the detection technics used by Old depended on cytotoxicity, the presence of circulating antibodies which were not cytotoxic could not have been detected.

Circulating antitumor antibodies have been identified in the serum of human cancer patients by Graham and Graham (11) and by Blakemore and McKenna (4). Fluorescent antibody studies were not performed by either of these groups. It is of considerable interest that Graham and Graham (11) were able to detect circulating antibodies in a sizable number of patients only after their primary tumors had been surgically excised. This agrees with the findings of the present study in which antibodies were demonstrable only after removal of the primary tumor. Finney et al. (8) have also detected circulating antitumor antibodies in human patients by tagged red cell hemagglutination and demonstrated a rise in titer when these patients were immunized with homogenates of their own tumor. Concentrated plasma globulin from these patients was found to be cytotoxic to autochthonous tumor cells, but fluorescent antibody studies were not performed.

The significance of these antibodies and their exact role in anti-tumor immunity is difficult to assess. Finney was able to correlate rises in antibody titer with slight but objective improvement in the course of the patient’s disease in some cases, while in other cases he was not. In the studies of Graham and Graham (11) and Blakemore and McKenna (4), the presence of antitumor antibody had essentially no demonstrable oncolytic effect. De Carvalho (6) has treated several cancer patients with an antitumor serum prepared in horses, and he reported some initially favorable responses. Green and Goldberg and their co-workers (10, 12, 13) have illustrated the cytotoxic action of heterologous antitumor antibody on cancer cells in the presence of complement. Bjorklund (3) developed heterologous antisera in horses that were cytotoxic to a number of human cancer cells, but not to normal human cells. Many other investigators have documented the fact that heterologous antitumor sera have cytolytic effect on tumor cells. Although such detailed studies have not as yet been performed with isologous or autochthonous antisera, it is likely...
that the isoantibodies in these systems are also cytotoxic to
cancer cells.

Why then the disproportion between the degree of immunity
discernible and the level of circulating antibodies? A number of
Finney's patients had very high antibody titers but onecytosis
was not dramatic. Conversely, in the present study very signif-
ificant immunity was elicited, but antibody titers were low. Sev-
eral possibilities suggest themselves. The cytotoxic action of anti-
body is a quantitative one. In human patients with grossly evi-
dent tumor masses, the number of tumor cells to be destroyed
may be too large or vascularity inadequate for proper delivery
of antibody to the cancer cells. In this study the challenge tumor
cell inocula consisted of only 1000 tumor cells. It may be that
the low antibody titers observed were sufficient to destroy 1000
cells but would not have been effective if a solid tumor mass of
several hundred million cells were present. An alternate possi-
bility is that tumor immunity is based on predominantly cellular
mechanisms, and that circulating antibodies are an incidental
finding having no direct correlation with the degree of immunity
observed.

The fact that no circulating antibody was detectable unless
the primary tumor was removed deserves comment. Isolated
instances of spontaneous regression of metastases following the
removal of primary tumors in man have been reported (2, 18,15).
Everson and Cole (7) reported 47 cases (culled from the world
literature) in which spontaneous regression of human malignant
tumors have been documented. Many of these patients had un-
degone partial or complete removal of their primary tumors
prior to the onset of tumor regression. However, antitumor anti-
body studies were not performed in any of these cases, and their
role, if any, in the observed tumor regression is therefore entirely
unknown. The role of the primary tumor in suppressing the
immune response remains unclear. If it acts by adsorbing circu-
lating antibody, thereby removing it from the circulation, this
fact could not be demonstrated in the present study by fluores-
cent antibody techniques. Some of the patients reported by Finney
et al. (8) had high antibody titers in the presence of gross tumor
masses; however, these antibodies usually produced little clinical
effect.

In the present study, circulating antitumor antibodies were
demonstrated in low titer in every experimental group of animals
in which increased host resistance to a subsequent isologous
tumor transplant was observed. These antibodies were not
demonstrable in animals that evidenced no such increase in
resistance. These antibodies reacted only with antigenic material
from the specific immunizing tumor and failed to react with
material from an unrelated tumor. Whether the antibodies
detected are cytotoxic or not has not as yet been determined.
Although a correlation may exist between specific antitumor
immunity and these circulating antibodies, more study is required
to establish this thesis and to elucidate the mechanisms involved.
It may be that the immunity observed was, in part, cell-mediated
in addition to being mediated by humoral antibody, or this
immunity may be entirely cell-mediated and the presence of
circulating antibody purely coincidental, bearing no causal
relationship to the immunity produced. Since the antibodies
detected were present in low titer, these last 2 hypotheses seem
attractive. Finally, much further study in additional tumor host
systems is needed to determine whether or not the presence of
circulating antitumor antibody invariably accompanies clinically
manifest tumor immunity.

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