Induction of Active Immunity against Isologous Tumor by in Vivo Conjugation of Heterologous Serum Protein with Autolysing Tumor Tissue

— A PRELIMINARY REPORT —

by

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Received for publication Oct. 31, 1963

Despite numerous and painstaking works, attempts to induce active immunity against autochthonous tumor have not been succeeded.[1)2)3)8)19)26)] The negative results have been regarded as a proof that spontaneous tumor has no antigenic distinction from the animal in which it originated.[7)]

On the other hand, papers continue to appear which claim that heterologous antiserum, produced against tumor tissue, contains antibodies directed specifically against the tumor but not the normal tissue.[9)14)17)18)28)] *In vitro* studies revealed that some of these antibodies were directed specifically against alcohol or chloroform extract of tumor tissue.[29)] Immunochemical analysis of such extracts proved that antigen in question was lipid.[4)23)24)25]

The tumor specific lipid, if such does exist, cannot induce immunological reaction in the original or isologous host, because the lipid is a hapten which is not antigenic unless combined with heterologous carrier protein.

The present study is designed to show if a spontaneous tumor of recent origin can produce tumor immunity in an isologous host when combined with heterologous carrier protein *in vivo*. Cross-immunity between two adenocarcinomas of different origin was also investigated.

METHODS AND RESULTS

The first series of experiments was designed to produce isologous immunity against a spontaneous tumor in mice of the inbred strain in which the tumor originated and also to obtain evidence indicating that the immunity is not dependent upon genetic difference between the tumor and the mice used in the experiment.

Mice of C3H/He line supplied by Kyoto University Inbred Animal Center were employed throughout the study. The mice were one to two months old and were fed *ad libitum* on a diet of Oriental Solid Chow and water. Mice of both sexes were approximately evenly divided among experimental and control groups. A mammary adenocarcinoma, Ca II, which arose spontaneously in a C3H female mouse was excised, and a small fragment was transplanted with an 18-gauge trocar in the subcutaneous tissues of the right side of the abdomen of fifteen mice of the same inbred strain. The first-transplant-generation tumor grew in eleven of the mice. Eighteen days after the transplantation, when the tumor attained a size of approximately 0.5 cm in diameter, nine of the tumor-bearing

mice were divided into two groups and subjected to the following manipulations. The first group of four mice received tumor ligation following technique described by Lewis[12)13)] and by Foley[2)] with some modification. The tumor was lifted free of the muscles underlying it and ligated by drawing a surgical thread around the base of the tumor. Thus, the tumor was deprived of blood supply and eventually autolysed. The second group of five mice also received tumor ligation in an identical manner, and at the same time 0.1ml of human serum obtained from an apparently healthy individual was injected into the ligated tumor. Both groups had their ligatures released after 48 hours. The autolysed content was absorbed within 48 hours and dry crust resulted. Five days after the ligation was released, each animal was tested for tumor immunity by subcutaneously implanting a second fragment of the tumor which filled an 18-gauge trocar for about 1mm of its length. The second, or challenge implant was obtained from mice bearing first transplant generation of the same tumor. The challenge implant was placed on the left side of the abdomen to avoid confusion with possible recurrence of the ligated tumor. The growth of tumor from the challenge implant was daily checked and mice which developed no tumor after a 60-day period of observation were recorded as negative tumor growth. Similar experiment was also carried out with the second to fifth transplant generation of the same tumor. Challenge was always made with the tumor of the same transplant generation as the immunizing tumor.

Table 1. Results of Attempts to Produce Isologous Immunity to a Transplanted Mammary Adenocarcinoma, Ca II, of Spontaneous Origin.

	Serum injection into ligated tumor			Simple tumor ligation			Untreated control		
#	M	T	%	M	T	%	M	T	%
1	5	3	60.0	4	4	100.0	25	19	76.0
2	8	5	62.5	8	5	62.5	40	34	85.0
3	15	7	46.6	10	8	80.0	40	29	72.5
4	15	8	53.3	15	10	66.6	40	35	87.5
5	15	5	33.3	15	11	73.3	40	33	82.5
Total	58	28	48.2	52	38	73.1	185	150	81.0

Abbreviations : # = Transplant generation of the tumor used for immunization and challenge. M = Number of mice challenged with tumor fragments. T = Number of tumor growths

Results given in Table 1 show that isologous tumor immunity was produced by injection of heterologous serum into ligated tumor. Tumor developed only in 48.2% of the mice which had received such a procedure. On the other hand, simple tumor ligation did not give any appreciable protection against growth of the challenge tumors. 73.1% of the mice which received tumor ligation developed tumors, whereas tumors grew in 81.0% of the untreated control mice. This indicates that the tumor and the animals are highly isologous and, therefore, the immunity observed in the experimental group does not largely depend on homograft reaction.

Evidence indicating that the tumor immunity was not dependent upon antibodies produced against the human serum was obtained in the second series of experiments,

whose results are given in Table 2. Skin pouches were made on the abdomen of mice, which were not bearing tumors, by stretching the skin outwards and ligating it. 0.1ml of the human serum was injected into the pouch. The ligation was released after 48 hours, and five days thereafter, the mice were challenged with the tumor implants. The tumors were accepted in 85.0% of the mice. This experiment indicates that the human serum alone cannot immunize the mice against subsequent tumor growth. It might be argued that the immunity produced either by injection of human serum or tumor ligation is too slight to be detected by a crude method of challenge inoculation, but their co-ordinated effects could be significant enough to account for the immunity observed following serum injection into ligated tumor. Five tumor-bearing mice had the tumors ligated and 0.1ml of the human serum was injected not into the ligated tumor but into a skin pouch made on the chest of the same mouse approximately 1 cm apart from the tumor. Ligatures were released 48 hours later. Challenge tumor grew in all mice. It is clear from the results that the direct contact of the human serum with the autolysing tumor tissue is inevitable in inducing the tumor immunity.

Table 2. Effects of Heterologous Serum Injection on the Growth of Subsequent Challenge Inoculation of Ca II.

	Serum injection into skin pouch			Serum injection into skin pouch and tumor ligation			Untreated control		
#	M	T	%	M	T	%	M	T	%
2	10	9	90.0	—	—	—	40	34	85.0
3	10	8	80.0	5	5	100.0	40	29	72.5
Total	20	17	85.0	5	5	100.0	80	63	78.7

All abbreviations are same as in Table 1.

In the first series of experiments, a relatively small number of the animals were tested for immunity against the tumor of early transplant generations. As a matter of fact, only thirteen out of fifty-eight mice bore the first and second-transplant-generation tumors. Because genetic deviation between host and tumor can increase during serial tumor transfer,[7)19)] the result of the experiments may have been influenced by genetic changes which ensued during five generations of serial transfer.

The third series of experiments was performed with another mammary adenocarcinoma, Ca III, which also originated spontaneously in a C3H female mouse. The mouse belonged to the same generation of the inbred strain as did the mouse in which Ca II originated. The original tumor was excised and transplanted to twenty-five C3H mice and eighteen developed tumors. Fifteen of them received serum injection into ligated tumor and were challenged with the tumor of the first transplant generation. Twenty-five mice received the same immunization procedure with tumor of the second transplant generation and were challenged with the tumor of the same generation. Results are given in Table 3. 75.0% of untreated control mice developed tumors, whereas tumors grew in only 45.0% of the immunized animals. The results indicate more strongly that genetic deviation which may have developed between the tumor and the host during the serial transfer is not largely responsible for the tumor immunity observed in the first series of the experiments.

Table 3. Results of Attempts to Produce Isologous Immunity to a Mammary Adenocarcinoma, Ca III, of its First and Second Transplant Generations.

	Serum injection into ligated tumor			Untreated control		
#	M	T	%	M	T	%
1	15	8	53.3	40	28	70.0
2	25	10	40.0	40	32	80.0
Total	40	18	45.0	80	60	75.0

All abbreviations are same as in Table 1.

The fourth series of experiments was designed to know if there is cross-immunity between two adenocarcinomas, Ca II and Ca III. Thirty C3H mice bearing Ca II of the sixth generation received tumor ligation and serum injection. One week later, the mice were challenged with Ca III of the third generation. As shown in Table 4, seventeen or 56.6% of the mice developed tumors. Conversely, twenty-five mice were immunized with the third generation Ca III and challenged with the sixth generation Ca II. Fourteen or 56.0% of the mice developed tumors. This indicates that there exists at least partial cross-immunity between Ca II and Ca III.

Table 4. Cross-immunity between Two Mammary Adenocarcinomas, Ca II and Ca III.

Number and generation of the tumors used for:		Serum injection into ligated tumor			Untreated control		
Immunization	Challenge	M	T	%	M	T	%
Ca II, 6th	Ca III, 3rd	30	17	56.6	10	7	70.0
Ca III, 3rd	Ca II, 6th	25	14	56.0	10	8	80.0

All abbreviations are same as in Table 1.

DISCUSSIONS

The experiments presented in this paper demonstrate that injection of heterologous serum into a ligated tumor can inhibit growth of subsequent challenge inoculation of the same tumor in an isologous host.

Induction of active immunity against indigenous tumor of spontaneous and recent origin in a highly inbred strain has been unsuccessful.[1)2)3)8)19)26)] Occasionally reported positive results in seemingly isologous tumor-host system[5)6)12)20)27)] were attributed to genetic deviation between the tumor and the host which developed during long period of serial transfer[7)] or to residual heterogeneity among members of the inbred strain.[19)] The results of the present experiments were in agreement with the reports cited above, and simple ligation of spontaneous adenocarcinoma did not give any detectable immune reaction in isologous animals.

In spite of the negative results in active tumor immunity, studies of heterologous immune serum produced against tumor tissue continue to claim that the immune serum

contain antibodies which would react specifically against certain antigens of the tissue.[9)14)17)28)] There are also reports that even non-protein tumor fraction can produce antibodies specific to the tumor. WITEBSKY[29)] reported the existence of cancer specific antigens in alcohol soluble fraction of cancer tissue. Rapport showed that chloroform-ethanol extract of Murphy-Sturm rat lymphosarcoma and H. Ep ≒3 human epidermoid carcinoma react specifically with rabbit antisera produced against respective tumors.[21)22)] Immunochemical analysis of the extract proved that the antigen in question is lipid.[4)23)24)25)]

The discrepancy in the results of active and passive tumor immunity suggests that cancer specific antigens, if such do exist, are antigenic only to heterologous animals and because they are not observed as "not self" by isologous hosts, they cannot provoke immune reaction in them.

Landsteiner[10)11)] showed that a hapten such as lipid is not antigenic by itself but can produce antibodies specific to it when conjugated with heterologous protein as a carrier. He stated that a hapten-protein conjugate is just a loose compound which can be established by adsorption of the hapten on the protein by simply mixing the two substances.

The present study is based on a working hypothesis that when heterologous serum is injected into tumor tissue, tumor specific hapten which may exist in the tissue will be conjugated with the serum and provoke tumor specific immune reaction in an isologous host. Mechanism of the tumor immunity observed in the present study is still unknown, but the results seem to support the validity of the working hypothesis presented above. In order to substantiate the hypothesis, it will be necessary to isolate tumor haptens and to study the immune reaction they may produce in isologous hosts when conjugated with heterologous carrier protein.

Exclusion of immunogenetic difference between tumor and host is the most important problem in the study of tumor immunity. Otherwise, the result will be largely influenced by homograft reaction and immune reaction observed cannot be regarded as directed specifically against the tumor. Homograft reaction cannot be completely excluded by simply employing an inbred strain of animals and a tumor which originated spontaneously in that particular strain. A tumor has ample opportunity to mutate during repeated transplantations, and may no longer be isologous to the recipient animals of the same strain[7)]. In the present study, genetic difference between tumor and animal was minimized by employing tumors of very early transplant generation and a highly inbred strain of animals. Yet, it cannot be claimed that the possibility of genetic difference and its consequent result of homograft reaction were completely ruled out from the present experiments. However, the observation that there was no significant difference in the tumor incidence between untreated control and the mice that had simple tumor ligation, provides a strong evidence that immunogenetic difference between the tumor and the mice was negligibly small.

The present study was also intended to give an experimental basis for active immunochemotherapy: the passive phase of which has been reported already.[26)] The term "immunochemotherapy," which was proposed in the preceding paper, denotes cancer chemotherapy in animals which are under the influence of induced immunological reaction. The immunity disclosed in the present study appears to be operative only in preventing

progressive growth of tumor tissue implanted subsequent to the immunization: tumors implanted previously were not adversely affected, as was occasionally observed with local recurrence of the ligated tumor. Combination of the active immunity and chemotherapy, i. e., active immunochemotherapy, can be expected, in view of the striking effects of passive immunochemotherapy presented in the preceding paper,[26] to produce better therapeutic results on well-established tumors.

The question that tumor immunity, observed in this study, is common to mammary adenocarcinomas originated in different mice of the same inbred strain is not yet fully answered, but at least partial cross-immunity was observed between the two tumors employed in this study.

SUMMARY

Two mammary adenocarcinomas, Ca II and Ca III, which arose spontaneously in C3H mice were excised and transplanted to mice of the same inbred strain. A tumor which grew in subcutaneous tissue of the mouse was ligated and deprived of blood supply. Immediately after the ligature was established, 0.1ml of human serum was injected into the tumor tissue. The ligature was released after 48 hours and autolysed content was eventually absorbed leaving a dry crust. Five days after release of the ligature, the mouse was challenged with the same tumor. More than 50% of the mice that had such a procedure did not develop the challenge tumor. Tumor ligation alone did not produce tumor immunity. Serum injection alone or tumor ligation and injection of the serum at a site away from the tumor also showed no tumor inhibition. Direct contact of the serum with the autolysing tumor tissue seems inevitable in inducing the tumor immunity. Homograft reaction was carefully avoided all through the experiments. A partial cross-immunity was observed between Ca II and Ca III.

The author wishes to express sincere gratitude to Professor CHISATO ARAKI and Assistant Professor IKUZO YOKOYAMA, both of the 1st Surgical Division Kyoto University Medical School, for their continuous guidance and encouragement.

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和文抄録

C3H系マウス自然発生乳癌の自動免疫

——血流遮断により自家融解した腫瘍組織に異種血清を混合し，混合物の吸収後に生じた抗腫瘍移植性——

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腫瘍の自動免疫の研究に際して最も重要な条件は，腫瘍と実験動物の間に免疫遺伝学的差異が無いことである．この条件を満たさない実験に見られる抗移植性は同種移植反応を包含しているので，腫瘍に特異的な免疫反応とは断じ難い．

同種移植反応を充分に除外するように計画された実験の成績は総て腫瘍に対する自動免疫反応の存在を否定している．一方腫瘍組織に対する異種免疫血清の研究の多くは腫瘍特異性抗原の存在を肯定する結果を報告している．この二つの一見矛盾したように見える結果の解釈として，異種動物で抗体を産生するような腫瘍特異性抗原が存在するとしても，それらの抗原は autologous or isologous host には "not self" として認識されないので，このような動物では免疫反応を起こすことが出来ないと考えるのが適当であろう．

Rapport, Witebsky 等は腫瘍組織のリピド或いはリポイド分劃に腫瘍特異性抗原が存在することを示している．又 Landsteiner は，リピドの如きそれ自体では抗原性を持たない hapten に異種血清を結合すると抗原性を獲得しその hapten に特異的に反応する抗体を産生することを示している．

この実験は腫瘍組織に異種血清を注入し，かつその腫瘍組織を自家融解吸収させることによつて抗腫瘍移植性を発生させ得るかを検したものであり，腫瘍組織中に存在すると考えられる腫瘍特異性 hapten に異種血清を結合すれば isologous host で免疫反応を起こし得るであろうと云う作業仮設に基いている．

C3Hマウスに自然発生した乳癌を同系のマウスに移植し，かつ実験の大部分を移植五代以内に，特にその内の多数は移植一，二代の腫瘍で行い，同種移植反応による artefact を可及的に除外した．

マウスの皮下に発育した腫瘍を，それを被う皮膚と共に結紮して血流を遮断すると，腫瘍は48時間以内に自家融解する．結紮を行つた直後．腫瘍組織内に人血清を注入し，in vivo で両者を接触させる．48時間後に結紮を解除すると内容物は数日中に吸収される．かゝる処理を行つたマウスに結紮後一週間目に同じ腫瘍を再移植すると，半数以上のマウスに抗移植性が認められ，腫瘍は発育しない．腫瘍を結紮しただけて，人血清を注入しない場合には抗移植性は発生しない．人血清の注射のみ，或いは腫瘍を結紮してそのマウスの他の部位に血清を注射しても抗移植性は見られない．自家融解した腫瘍組織に人血清が直接接触することが，抗移植性の発生に不可欠な条件であると考えられる．

尚，腫瘍の結紮のみを行つた群と未処置群の間に移植腫瘍の発生率に有意の差は認められなかつたが，この事実は実験に用いた腫瘍とマウスの間に免疫遺伝学的差違が極めて少なく，同種移植反応の介入する余地が殆んどないことを示している．しかし移植腫瘍を用いた実験である以上，同種移植反応を完全に除外することは理論上不可能であるから，この実験で観察された抗移植性が腫瘍特異的であると断定するには，尚問題が残されている．

C3Hマウスから分離継代移植した二種の自然発生乳癌の間に，或る程度の交叉免疫が成立することも認められた．

この実験は同種移植免疫反応を充分に除外した上で，自然発生癌に対する抗移植性を実験的に発生させ得た最初のものと思われる．抗移植性の発生機序はこの実験では解明するに至らなかつたが，実験結果は前述の作業仮設を支持するものと思われる．この点は更に今後，腫瘍組織から分離精製した haptenを異種血清と混合し，その混合物で免疫感作を行い抗移植性の発生を検することによつて明らかにされよう．

又この実験で見られた抗移植性が腫瘍特異的であるとしても，それが腫瘍細胞自体に向けられたものであるか，或いはマウス乳癌のウイルス性発癌因子に対するものであるかは今後の検討を要する問題である．