STUDIES ON ANTI-TUMOR IHMUNITY I. HOMOLOGOUS IMMUNITY AGINST EHRLICH ASCITES TUMOR (ACQUIRED TYPE OF IMMUNITY AGAINST TUMOR)

Momoe Soeda
(IInd Research Institute, Technical R. and D.H.Q., J.D.A.)

腫瘍免疫に関する研究 I. Ehrlich ascites tumor に対する同種性免疫

防衛庁技術本部第2研究所
添田百枝

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intact 又は不活化した Ehrlich ascitis tumor 細胞を DDS-系マウスの腹腔内に接種した際のマウスの免疫的 response を研究し，高度の感受性を持つ DDS-系マウスが Ehrlich ascites tumor に対し不感受性化する際の基礎的メカニズムの解明に貢献し得るデーターを得んとした．

加熱又は超音波により不活化したＥＡＣ細胞を腹腔内に接種してもマウスは発病しないが，併しこれによりマウスは対腫瘍免疫を獲得して，致死量を遙かに越える intact-EAC 細胞の接種にも耐えるようになる．一方ＥＡＣマウス腹腔液の2,000 r.p.m. 30分遠心上清（ＥＡＣ細胞 frce）を化学的に濃縮処理した preparation をワクチンとしてマウスに接種しても同じ効果が得られる．既に1961年著者は Ehrlich ascites tumor がＥＡＣマウス腹水の 4,000～20,000 r.p.m.30分遠沈上清の接種により発生することを公表したが，これと併せ考えるとマウスの対ＥＡＣ免疫 response は腫瘍細胞自体に指向されるよりも寧ろ著者の云う Carcinogenic agent に向けられると考え度い．

Synopsis

Immunological response of DDS mice to inoculation of Ehrlich ascites tumor cells, intact or inactivated by exposure to heat or ultrasonic waves, was experimentally studied in order to contribute to some extension of knowledge about the fundamental mechanism by which highly susceptible animals acquire a certain degree of anti-tumor immunity and become resistant against a dose of tumor cells intolerable for usual normal animals. Tumor cells inactivated by exposure to heat or ultrasonic waves were incapable of inducing tumor in animals, but such treated mice proved to be endowed with a relatively high anti-tumor resistance and well tolerated subsequent implantation of by far a larger dose of tumor cells.

Concentrated preparation of cell-free ascites fluid of EAC mice also proved capable of inducing antitumor resistance in mice when used as a vaccine, the degree of which appeared to be higher than that induced by tumor cell-vaccine. As previously reported in 1961, we found that the supernatant of ascites fluid, entirely freed from EAC cells by centrifugation at 20,000 r.p.m. for 30 minutes, proved still capable of inducing typical ascites tumor in DDS mice. From these experimetal evidences it seems reasonable to consider that some agent found in cell-free ascites would be responsible for malignant transformation of host's cells, and that immunological response of the recipient may not be necessarily directed toward intact cells as a whole, but rather towards such agent present not only within the tumor cells but also in ascites fluid of EAC mice.

Introduction

Since 1959, we have studied on transplantability of Ehrlich ascites tumor cells into mice of DDS strain and found the fact that an ascites tumor inducing agent was present not only within cells but also in ascites fluid in which cells being immersed. According to various evidences obtained from a series of experimental studies on immunity against malignant tumors of animals we have postulated that this agent may be of ultramicroscopic size, because it could not be removed even if ultracentrifuged at 20,000 r.p.m. for 30 minutes. We called it, tentatively, "carcinogenic agent of Ehrlich ascites carcinoma"[9]. On this agent, Soeda et al. reported at the general meeting of Japanese Bacteriological Association in 1961.

Subsequently we have succeeded in photographing this agent in the form of almost uniform virus-like particles, with the help of Dr. Kimura of Central Institute of Hitachi Co. The detail of this finding was reported at the 147th Scientific Conference of Japanese Antibiotic Association in 1962[12].

Recently we have carried out studies on experimentally induced resistance of mice ageinst Ehrlich ascites carcinoma (EAC) by vaccination with inactivated or intact EAC cells or with concentrated preparation of the carcinogenic agent of EAC, as an important step in the course of elucidation of the fundamental mechanism by which an acquired type of anti-tumor resistance of mice being induced. From the results of these studies we have presumed that an active immunological process would play an important role in the acquired type of anti-tumor resistance of mice, and that such immunological response of hosts would not be directed toward tumor cells as a whole, but rather toward the carcinogenic agent existing in both intraand extra-cellular fluids of tumor cells.

In this paper I will report on the experimental results obtained from recent studies concerning the following five subjects.

1) Relationship between graded doses of EAC cells given i.p. into mice and the survival rate of inoculated mice.

2) Alteration of transplantability of EAC cells by application of heat.

3) Alteration of transplantability of EAC cells by exposure to ultrasonic waves.

4) Concentrated preparation of the carcinogenic agent obtained from both intra- and extra-cellular fluids of EAC cells.

5) The acquired type of anti-EAC resistance of mice induced by vaccination with inactive and intact tumor cells or with concentrated preparation of the carcinogenic agent of EAC cells.

These studies were done in order to contribute to some extention of knowledge about the fundamental

mechanism by which highly susceptible mice of DDS strain acquire a certain degree of anti-EAC resistance and become immune against challenge with by far a larger dose of EAC cells.

Materials and Methods

Animals: Male mice of DDS strain weighing about 20 g. were adopted for study. It has generally been accepted that susceptibility of mice to EAC is considerably variable from one strain to another. In 1959, I had tested on susceptibility of various strains then available, and found a substrain derived from the original DD strain, which proved to be highly susceptible to EAC, and this strain was named DDS strain by myself. Graded doses of EAC cells were inoculated i.p. into mice and we realized that in ordinary conditions 10^3 cells may be regarded as an absolutely fatal dose for DDS-mice.

EAC cells: EAC cells were maintained by serial inoculation into mice with 10^6 to 10^7 cells per mouse. Ascites fluid was collected from EAC-mice 2 to 3 weeks after inoculation and used for challege tests. EAC cell count of ascites fluid was done according to the ordinary method for the white cell count of the blood and thus a dilution containing 10^6 or 10^7 cells per ml. was prepared. For the studies on the influence of heat application and of exposure to ultrasonic waves to transplantability of EAC cells this dilution was used.

Concentrated preparation of the carcinogenic agent: The following procedures were performed. Ascites fluid was collected from EAC mice and centrifuged at 3,000 r.p.m. for 30 minutes, and the cell-free supernatant was removed. To this, 50% solution of zinc chloride was added at the rate of 1:50 to 100, and the developed precipitate was removed by centrifugation. This precipitate was then dissolved in 10% solution of disodium phosphate to obtain a partially concentrated dilution of the active agent (A fraction).

Then this fraction was mixed with an equal amount of methanol or ethanol and the resultant precipitate was removed by centrifugation (Precipitate 1).

The supernatant fluid of Precipitate 1. was again mixed with methanol and Precipitate 2 thus obtained, and the same procedure was once more repeated to get Precipitate 3.

Three precipitates were dissolved separately in distilled water and centrifuged at 3,000 r.p.m. for 30

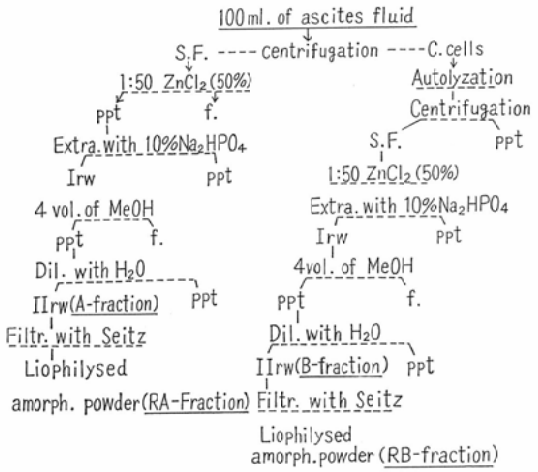

Table 1. Purification procedures of the carcinogenic agent in the ascites of EAC-mice.

minutes to remove insoluble fractions. Each solution was filtered through a steril Seitz filter and transferred to cellophane tubes to dialyze against water. The solution was again filtered and distributed to ampoules and freeze-dried (RA-1 fraction, RA-2 fraction and RA-3 fraction).

Similar procedures were applied to obtain concentrated preparation from intracellular fluid of EAC cells. Sedimented cells were subjected to autolyzation by keeping them with distilled water (1:5) at 37°C for one hour, and centrifuged at 3,000 r.p.m. for 30 minutes. B fraction, RB-1, RB-2 and RB-3 fractions were thus obtained.

Results

Exp. 1. Inactivation of EAC cells by exposure to heat

20 ml. of a dilution of ascites fluid of EAC mice, containing 10^6 to 10^7 cancer cells per ml., was equally divided into 4 test tubes. Tubes were kept at 40°C for 15 and 30 minutes or at 60°C for 15 and 30 minutes. The cells kept at 40°C for 15 minutes proved to maintain transplantability and capable of inducing EAC in mice, while those kept at 40°C for 30 minutes or at 60°C for 15 to 30 minutes were inactivated and proved incapable of inducing EAC in mice. However, the mice inoculated with 10^5 to 10^6 EAC cells inactivated by exposure to heat proved to be endowed with a certain degree of anti-EAC resistance and most of them tolerated the 2nd challenge test with 10^4 intact cancer cells, a dose ten times as large as an absolutely fatal dose for usual DDS mice.

Exp. 2. Inactivation of EAC cells by exposure to ultrasonic waves

10 ml. of the same dilution was equally divided into 2 tubes. Tubes were exposed to ultrasonic waves generated by a sonic oscillator, Kubota Co., with a output power of 200 W and a frequency of 10 Kc/sec. The first tube was exposed for 15 minutes, while the second one for 30 minutes.

Mice were given i.p. 10^5 or 10^6 EAC cells thus exposed to ultrasonic waves. The cells in the first tube maintained transplantability and proved capable of inducing EAC in mice. All mice developed typical EAC and died within 30 days. On the contrary, the cells in the second tube were inactivated and proved incapable of inducing EAC in mice. Such mice also became resistant against EAC and most mice tolerated the 2nd challenge test with 10^4 intact cancer cells.

Exp. 3. Relationship between graded doses of EAC cells and the survival rate of inoculated mice

Ascite fluid was collected from EAC mice between the 10th and 20th day of inoculation with 10^6 to 10^7 EAC cells and serially diluted with saline solution to obtain several dilutions containing EAC cells in the range from 10^2 to 10^6 per 0.1 ml.

A single cell inoculated i.p. into mice was incapable of inducing EAC, but 10 cells seemed sufficient to induce EAC in half the cases of DDS mice, while with a dsoe of 10^2 cells almost all mice appeared difficult to avoid deaths from developing cancer. A dose of 10^3 cells was regarded as an absolutely fatal dose for usual mice of DDS strain (Table 2).

With 10^4 cells, however, one exceptional mouse was found among 10 mice. This may be an exceptional case found very rarely among so many mice of DDS strain, and may be regarded as a naturally immune mouse against EAC.

Exp. 4. Acquired anti-tumor resistance induced by vaccination with EAC cells inactivated by exposure to heat or ultrasonic waves as well as with increasing doses of intact EAC cells

As mentioned previously, all mice survived when they were given i.p. 10^5 to 10^6 EAC cells inactivated

Table 2. Relationship between EAC cell count and the outcome of mice

Inoculation dose of EAC cells	Number of used animals	Typical deaths	Number of survived animals	Survival rate
10^0	6	0	6	6/ 6
10^1	6	3	3	3/ 6
10^2	10	9	1	1/10
10^3	10	10	0	0
10^4	10	9	1*	1/10
10^5	10	10	0	0

* naturally immune mouse rarely found among so many mice

either by keeping at 40°C for 30 minutes or at 60°C for 15 to 30 minutes or by exposure to ultrasonic waves for 30 minutes. Such mice were divided into 2 groups. Mice in the 1st group were given i.p. 10^5 intact cancer cells, while those of the 2nd group were challenged with 10^4 intact cancer cells. Ten mice in the 1st group all developed typical EAC and died within 30 days, while 8 of 10 mice in the 2nd group never develop typical EAC and survived. These 8 mice were again inoculated with 10^5 intact cancer cells, and 6 mice tolerated this challenge test. Furthermore, they were challenged with 10^6 intact cells and 3 mice survived without any signs characteristic of EAC.

Thus, by repeated inoculation with inactivated EAC cells and then with a tolerable dose of EAC cells, mice of DDS strain can be made immune against a dose introlerable for normal mice. It is also of interest that most mice pretreated with inactivated EAC cells can well tolerate more than 10 times as large as an absolutely fatal dose for usual mice of DDS strain.

Exp. 5. Rechallenge experiment to a naturally immune mouse

As mentioned in Exp. 3, one exceptional mouse was found among 10 mice inoculated with 10^4 intact cancer cells. This mouse was regarded as a naturally immune case very rarely found among so many mice of DDS strain. About 10 weeks later this mouse was challenged with 10^5 cancer cells, but this showed no characteristic signs until 17th day, when the third inoculation with 10^5 cancer cells was done. The mouse survived without any manifestations of EAC up to 144th day, when it was killed and specimens of tissues and organs were removed for histological examination, which failed to reveal tumor.

This case was completly immune against 10^4 EAC cells from the beginning and further proved insusceptible to the following two challenges with 10^5 and 10^6 cancer cells respectively.

At the present time it is not precisely known whether the natural type of anti-tumor resistance, as observed in this case, is identical in nature with the acquired type of anti-tumor resistance or not.

Exp. 6. Protection of mice against EAC by intraperitoneal vaccination of A fraction

Mice were i.p. vaccinated with graded doses of this fraction and 6 days later they were challenged with 10^5 cancer cells. With 0.1 ml., 2 of 3 mice developed typical EAC and died on the 39th and 63rd day respectively, but another mouse survived for 85 days without any manifestations of EAC. With 0.25 ml., 2 of 3 mice suffered from EAC and died on the 52nd and 53rd day respectively, but another mouse survived without typical signs up to the 69th day, when it was killed and histologically examined. No characteristic histological findings were revealed.

With 0.5 ml., 2 f 4 mice survived without developing EAC and on the 69th day it was killed and histological examination was done, which failed to reveal characteristic findings. Two mice did develop typical

Table 3. Rechalldnge to a naturally immune mouse.

Dose of 1st challenge	Dose of 2nd (after 73 chal. days)		Dose of 3rd (after 90 chal. days)		Survival or (on 144th Death day)
10^4*	10^5 (tolerated)		10^6 (tolerated)		n.c. **(alive)
Control (1)	1 10^5 dead on 24th				
	2 〃 〃 20				
	3 〃 〃 23				
	Control (2)	4	10^6 dead on	16th	
		5	〃 〃	21st	
		6	〃 〃	21	
	Control (3)	7	10^5 dead on	36th	
		8	〃 〃	36	
		9	〃 〃	40	
	Control (4)	10	10^4 dead on	36th	
		11	〃 〃	24	
		12	〃 〃	16	

* This mouse was found in Exp. 3. **means not cancer.

Table 4. Protection of mice against EAC by intraperitoneal vaccination of A-fraction.

No. of used mice	Dose of A-fraction	Chal. dose of EACC	Typicol death on	Survival rate	2nd chal. after 69 days	Typical death on
1	0.10ml.	10^5	38th			
2	0.10	〃	n.c.	1/ 3	10^6	n.c. (85th)
3	0.10	〃	63rd			
4	0.25ml.	10^5	n.c.			
5	0.25	〃	52nd	1/ 3	sacrificed (n.c.)	
6	0.25	〃	53rd			
7	0.50ml.	10^5	n.c.			
8	0.50	〃	30th	2/ 4		
9	0.50	〃	53rd			
10	0.50	〃	n.c.			
		11 10^5	21st			
	Control	12	24th	0		
		13	27th			

EAC and died on the 30th and 53rd day respectively. The results are shown in Table 4.

It should be pointed out that the average survival period of 6 test mice was 48 days, whereas that of 3 contolr mice was 24 days. The former was about 2 times as long as the latter.

The same test was repeated with another sample of A fraction.

Total 8 mice were divided into 2 groups. All mice were i.p. vaccinated with 0.5 ml. of A fraction, and 4 mice in the first group were challenged 7 days later with 10^5 intact cancer cells, while 4 mice in the second group were challenged in the same fashion 13 days later. 2 mice of the 1st gorup and one mouse of the 2nd group survived without developing cancer for 84 days.

Thus about a half of 12 mice vaccinated with 0.5 ml. of A fraction became immune against a challege dose of 10^5 EAC cells.

Exp. 7. Protection of mice against EAC by i.p. vaccination with RA fraction.

Mice were i.p. inoculated with 0.25 ml. or 0.5 ml of RA fraction and 5 days later they were challenged with 10^5 intact cancer cells. Among 4 mice vaccinated with 0.25 ml. 3 mice developed typical EAC and one died on the 15th day, but the other two survived on the 40th day. Another mouse did not show any

manifestations characteristic of EAC. Among 4 mice vaccinated with 0.5 ml. 2 mice died of typical EAC, while other 2 mice survived without showing any characteristic signs of the disezse. Three control mice inoculated with 10^5 intact acncer cells suffered from EAC and dild on the 26th, 28th and 32nd day respectively (Table 5).

Table 5. Protection of mice against EAC by intraperitoneal vaccination of RA-fraction.

No. of used mice	Dose of RA-fra-ction	Chal. dose of EACC	Typical deaths on	Survival on 40 th day	Survival rate
1	0.25ml.	10^5		c. but alive	
2	0.25	〃		〃	1/ 4
3	0.25	〃		〃	
4	0.25	〃	n.c.	n.c.	
5	0.50ml.	〃	30th		
6	0.50	〃		c. but alive	2/ 4
7	0.50	〃	n.c.	n.c.	
8	0.50	〃	n.c.	n.c.	
	9	10^5	32nd		
	10	〃	28th		0
	11	〃	26th		

* 0 25ml. correspondsto about 2.0㎎ of RA-fraction ** 0.5 ml. corrasponds to about 4.0㎎ of RA-fraction.

Exp. 8. Protective effect of B fraction.

Mice were i.p. vaccinated with 0.25 ml. or 0.5 ml of B fraction and 5 days later they were challenged with 10^5 intact cancer cells. Each 4 mice in both groups could not escape deaths from typical EAC. Thus we could not recognize any protective effect of B fraction.

Exp. 9. Protection of mice against EAC by i.p. vaccination with A or B fraction after challenge with intact cancer cells.

Mice were challenged with 10^6 intact cancer cells and 1 day later a daily dose of 0.5 ml. of A or B fraction was i.p. injected for 4 succesive days. Two of 3 mice treated with A fraction were able to avoid deaths from EAC,while all 3 mice treated with B fraction died from typical EAC between the 16th and 33rd day (Table 6).

Table 6. Protection of mice by A- or B-fraction given after challege inoculation.

No. of mice	Challenge dose of EACC	Treatment for 4 days with Afrac.	B-frac.	Saline	Typical deaths on	Survival rate
1	10^6	0.50ml.			n.c.	
2	〃	0.50			n.c.	2/ 3
3	〃	0.50			26th	
4	〃		0.50ml.		18	
5	〃		0.50		16	0/ 3
6	〃		0.50		33rd	
7	〃			0.50ml.	27th	
8	〃			0.50	15th	0/ 3
9	〃			0.50	23rd	
Control	10 10^6				23rd	
	11 〃				18th	0/ 3
	12 〃				16th	

It was a somewhat unexpected finding that almost no protectiev effect was recognized in B fraction, because the effective principle would be expected to be contained in by far a larger contet within B frac-

tion in view of its method for preparation.

Discussion

Graded doses of EAC cells were i.p. inoculated into mice of DDS strain. A dose of 10^3 EAC cells was sufficient to kill almost all mice and this was regarded as an minimum fatal dose for usual miec of this strain. An exceptional mouse was found among mice given i.p. 10^4 cells, which may be a naturally immune mouse very rarely found among so many DDS mice.

Vaccination with inactivated or intact EAC cells proved papable of inducing a relatively high resistance against subsequent inoculation with a higher dose of intact cancer cells. Whether is this acquired type of resistance identical in nature with the natural type of resistance is open to some question.

Repeated vaccinations with increasing doses of inactivated or intact EAC cells proved to be capable of enhancing the degree of resistance of mice against ascites tumor, and therefore an active immunological process should play an important role in the fundamental mechanism by which mice could become more and more resistant against EAC. Recently, in experimental studies on the cytotoxic effect of heterologous spleen cells of immunized rabbits on the Landschutz asciges tumor cells, Stuart[10)11)] has assumed that the beneficial effect of treatment with such spleen cells may be due to anti-tumor antibody which being carried by intact immunized cells on their surface or interior, which, may enable the cells to adhere to tumor cells leading to cell damage following in vivo fixation of complement.

We have done a similar study on the effect fo spleen cells of immunized rabbits on Ehrlich ascites tumor cells and obtained the results in accord with those of Stuart[14)]. The detail of this study will be reported in the next paper. We have also studied on homologous spleen cells in the same fashion and recognized a similar beneficial effect of immunized intact cells of homologous animals. Whether or not the acquired type of anti-tumor resistance induced by repeated treatment of susceptible animals with tumor cells or modified ascites fraction is of the same nature as that induced by immunization of insusceptible animals with intact tumor cells is entirely unknown at the present time, because antigenic substance responsible for production of antibody related to acquired resistance or cytotoxic effect of lymphoid cells has not been identified yet. It seems, proboble however, that the acquired type of resistance developed in susceptible host would also be due to anti-tumor antibody carried by intact lymphoid cells, because spleen cells of immunized susceptile host exert a similar beneficial cytotoxic effect on tumor cells and confer some passive resistance to the recipients.

The natural type of resistance rarely found among so many mice of DDS strain may be, probably, due to the same mechanism, although experimental evidences for such case are extremely difficult to obtain. According to our experience a naturally immune mouse which well tolerated the initial challenge with 10^4 EAC cells proved insusceptible to subsequent inoculation of 10^5 and 10^6 tumor cells. It was killed 144 days after the initial challenge and its spleen cells were tested on their inhibitory action against tumor cells according to the procedures of Stuart. These spleen cells proved capable of inducing a considerably high anti-tumor resistance in mice. In this case, however, the mouse was inoculated with 10^4 EAC cells followed by inoculation with 10^5 and 10^6 tumor cells at intervals of 73 and 17 days respectively, so that whether such anti-tumor effect of spleen cells is of natural nature or not may be a very difficult problem. As Stuart has suggested, anti-tumor or cytotoxic effect of immunized lymphoid cells may play an important role in the mechanism by which highly susceptible mice of DDS strain being endowed with a

relatively high anti-tumor resistance, and this effect may be due to an anti-tumor antibody carried by intact immunized cells. The view that immunological response of animals immunized with intact or inactivated tumor cells would be directed to the tumor cells as a whole seems to be an oversimplification of a more complicated reaction. Experimental studies on various components of tumor cells to search for an antigenic fraction principally responsible for production of anti-tumor antibody have been carried out by many workers, but no success was achieved in obtaining satisfactory results.

As previously reported[9], we found that ascites fluid entirely freed from EAC cells by centrifugation even at 20,000 r.p.m. proved still capable of inducing typical ascites tumor in mice in almost the same fashion as intact EAC cells. According to this fact we have postulated that some agent in cell-free ascites must, at least, contribute to the actual transplantability of EAC cells into mice. On the other hand, as shown in this paper, concentrated preparation of such agent was prepared and tested on its value as a protective vaccine against EAC, and the vaccinated mice with such preparation proved to be endowed with a relatively high resistance against tumor and about a half of such mice could escape from deaths due to the typical acsites tumor following i.p. implantation of 10^5 intact tumor cells.

The reason why similar preparation obtained from intracellular fluid of EAC cells was almost of no value as a protective vaccine is entirely unknown at the present time.

From these evidences it seems reasonable to consider that some aggent presnet in both intra- and extra-cellular fluids of EAC cells intimatily relates to the transplantability of EAC cells and that the acquired type of anti-tumor resistance induced in mice may be regarded to develop as a result of an active immunological response of mice to this agent rather than to tumor cells as a whole.

Summary and Conclusion

1) Transplantability of EAC cells into mice was not lost by keeping at 40°C for 15 minutes, but inactivated by keeping at 40°C for 30 minutes or at 60°C for 15 minutes. Inactivated cells proved incapable of inducing EAC in mice but such mice acquired a certain degree of resistance against EAC and about 80 per cent of such mice proved insusceptible to inoculation of 10^4 intact EAC cells, a dose absolutely atal for normal mice of DDS strain.

2) EAC cells exposed to ultra-sonic waves for 15 to 30 minutes were also inactivated and became incapable of inducing EAC in mice even if a dose of 10^5 to 10^6 EAC cells was used as a challenge dose. Such mice, however, became endowed with a certain degree of resistance and most of them proved resistant against inoculation of 10^4 intact cancer cells.

3) A single EAC cell inoculated i.p. into mice was incapable of inducing EAC, but 10 cells seemed sufficient to induce EAC in half of DDS mice, while with a dose of 10^2 cells almost all mice appeared difficult to avoid deaths from developing cancer. A single dose of 10^3 cells was regarded as an absolutely fatal dose for usual mice of DDS strain.

4) An exceptional case was found among 10 mice given i.p. 10^4 EAC cells. This mouse also tolerated 2 successive challenges with 10^5 and 10^6 EAC cells done at intervals of 73 and 17 days respectively, and survived up to the 144th day, when it was killed and specimens of tissues and organs were removed for histological examination. A detailed postmortem with microscopic examination failed to reveal tumor. This may be regarded as a naturally immune mouse very rarely found among so many mice of DDS strain.

5) Mice vaccinated with 10^5 to 10^6 EAC cells inactivated by heat application or by exposure to ultra-

sonic waves could not tolerate a challenge test with 10^5 intact cells, but 8 of 10 such mice were able to pass safely throuh an initial challenge test with 10^4 intact cancer cells, and 6 mice proved insusceptible to the 2nd challenge with 10^5 intact cancer cells. Furthermore these 6 survived mice were again tested with 10^6 intact cancer cells and 3 mice survived. Thus by repeated vaccination with increasing doses of inactivated or intact cells anti-tumor resistance of mice seems apparently increase gradually, indicating that an active immunological process may play an important role in the fundamental mechanism by which highly susceptible mice of DDS strain can become more and more insusceptible to a larger dose of EAC cells.

6) Concentrated preparation of the carcinogenic agent was prepared from cellfree ascites fluid from EAC mice. This preparation proved capable of inducing anti-tumor resistance in mice when used as a vaccine 5 to 7 days prior to challenge with intact cancer cells. A single dose of 0.5 ml of this preparation given i.p. into mice saved 2 of 4 mice inoculated with 10^5 intact cancer cells, a dose about 100 times as arge as a minimum fatal dose for usual normal mice. Such a high effect could not be achieved by vaccination with 10^6 cells inactivated by exposure to heat or ultrasonic waves.

The same preparation was also tested on its anti-tumor activity when used posterior to challenge with intact cells. Daily dose of 0.5 ml. was given i.p. into mice, once a day, for 4 days starting 24 hours after chanllenge with 10^6 intact cancer cells, which prevented 2 out of 3 mice from developing cancer.

7) Similar preparation was prepared by the same procedure from intra-cellular fluid of EAC cells. This was tested on its anti-tumor activity when used prior and posterior to challenge with intact cancer cells. It was an unexpected finding that this preparation proved almost of no value as a protective vaccine and incapable of inducing anti-tumor resistance in mice.

The reason why the preparation obtained from intra-cellular fluid of cancer cells did prove almost of no value as a protective vaccine is not clear at the present time. However, it is of much interest that an active substance responsible for active immunization of mice appears to be present in by far a higher concentration in cell-free supernatant of ascites fluid of EAC mice.

We have done this study in order to contribute to some extension of knowledge about the fundamental mechanism by which the acquired type of antitumor resistance being induced when mice were inoculated with inactivated or intact tumor cells. Similar antitumor resistance of mice could be induced by vaccination with modified preparation of cell-free ascites fluid of EAC mice, the degree of which appeared higher than that induced by tumor cell-vaccine.

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