

# IMMUNOCHEMOTHERAPY OF CANCER

## EFFECTS OF ANTICANCER CHEMOTHERAPEUTIC AGENTS AGAINST EHRLICH ASCITES CARCINOMA IN C3H MICE IMMUNIZED WITH HETEROLOGOUS ANTICANCER SERUM

by

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The success of chemotherapy in infectious diseases has stimulated the development of chemotherapeutic agents against cancer. It is no exaggeration to say that some of these agents are as effective against certain neoplastic cells *in vitro* as are most of the antibiotics against bacterial cells. However, there is a considerable difference between infectious and neoplastic diseases in responsiveness to chemotherapy. The difference is mostly due to the fact that natural immune reactions of the host, which play a major role in bacterial chemotherapy, are inadequate or even lacking in the treatment of cancer. If immune reactions could be artificially induced or augmented, the outcome of cancer chemotherapy would be greatly changed.

Establishment of host defense against cancer has attracted the attention of many workers, and a variety of immunological procedures have been examined with experimental and human cancers. Active immunization of autochthonous cancer has thus far met with failure.<sup>13)16)17)29)55)58)</sup> Treatment of cancer with heterologous immune serum, on the other hand, has been reported to afford some prophylactic effect<sup>15)49)52)54)70)</sup> and to produce temporary tumor regressions.<sup>5)53)</sup>

Host defense reactions induced by present immunological methods have been disappointing in controlling cancer by themselves, but their usefulness as an adjunct to cancer chemotherapy has been gradually recognized. Chemotherapy of cancer in animals "immunized" either passively or actively, has recently been studied by several,<sup>34)63)68)72)</sup> and improvement of the therapeutic effect has been reported.

The purpose of this study is to demonstrate the effects of cancer chemotherapeutic agents in animals immunized with heterologous anticancer immune sera. Because the study was planned to provide an experimental basis for chemotherapy of human cancer in a late stage with massive metastasis, treatment of well-established animal tumors was included as an important part of the study. Experiments were also designed to determine if the effect of the anti-cancer immune serum is directed to antigens unique to the tumor.

### MATERIALS AND METHODS

Ehrlich ascites carcinoma (EAC) was employed in this study. EAC was maintained

by weekly intraperitoneal injection of 0.1ml of the ascites into C3H mouse.

Mice of C3H/He line supplied by Kyoto University Inbred Animal Center were used throughout the study. The mice were 6 to 10 weeks old, weighing 20 to 25 gm and were fed *ad libitum* on a diet of Oriental Solid Chow and water.

*Preparation of antigens used for immunization of rabbits, absorption of antisera and agglutination test :*

EAC was harvested aseptically from several mice one week after inoculation. Only non-hemorrhagic ascitic fluids were pooled and diluted twofold with 0.85% saline. The suspension was then centrifuged at 300 r. p. m. for one minute and EAC cells were separated from contaminating erythrocytes. The cells were washed four times in 0.85% saline by alternate re-suspension and centrifugation. The washed cells were suspended in 0.85% saline to make a 10% cell suspension. Mammary gland, liver, and kidney removed aseptically from normal C3H mice were homogenized by pressing through a 180 mesh stainless steel screen, washed and prepared as a 10% suspension in the same way as EAC cells. A 10% erythrocyte suspension was also prepared in a similar manner. Equal portions of the normal cell suspensions (mammary gland, liver, kidney and erythrocyte) were mixed together to make a "mixed normal cell suspension."

*Production of antisera :*

Ten rabbits were used for the production of antiserum against EAC. Immediately before the immunization, 10 ml of blood was withdrawn from each rabbit; serum was separated, pooled and stored in a deep-freezer to serve as a normal control serum. Each rabbit received 5 ml of freshly prepared EAC suspension; i. e., 3 ml intravenously and 2 ml intradermally. The injection was repeated every other day over a period of 9 days. Seven days after the fifth injection, all rabbits were exsanguinated by cardiac puncture and serum was separated by centrifugation. Agglutination test was performed on the serum specimens and four of them showed anti-EAC titer exceeding 2,560. These sera were pooled and stored in a deep-freezer. Others were discarded. Antiserum was also produced in five rabbits against "mixed normal cell suspension" in the same immunization schedule. Agglutination test revealed that anti-EAC titers of the five serum specimens were 640 to 1,280. All sera were pooled and stored in a deep-freezer.

*Absorption of antisera with normal mouse cells :*

The "mixed normal cell suspension" was centrifuged at 3,000 r. p. m. for 15 minutes and the supernatant was discarded. The packed cells were mixed with antiserum in the proportion of 1:10. The mixture was shaken well at room temperature for one hour and kept cold over night. The cells were removed by centrifugation. The absorption was repeated three times. Unabsorbed antisera were routinely used in the experiments, unless otherwise stated.

*Agglutination test :*

Test antiserum was diluted ten-fold and then serially diluted two-fold in a series of twelve test tubes. A drop of test antigen suspension was added to each test tube and mixed well with the diluted antiserum. The test tubes were left at room temperature for an hour and then were kept in an ice-box. Final readings were made at the 24th hour.

*Techniques of treatments :*

Ascitic fluid was aspirated from a C3H mouse 5 to 7 days after inoculation of EAC. The fluid was diluted tenfold, 0.1ml of which was inoculated intraperitoneally into each C3H mouse. Such inoculata contained approximately one million EAC cells. In most of the experiments reported here, treatment was given only once 48 hours after the inoculation by intraperitoneal injection of antiserum and/or anticancer chemotherapeutic agent. All the antisera and normal control rabbit serum used in the experimental treatments were inactivated at 56°C for 30 minutes. Any treatments other than this will be individually described.

RESULTS

Table 1 shows *in vitro* agglutination titers of two antisera and normal rabbit serum (NRS) against EAC and normal mouse tissues. Normal C3H mouse liver cells and erythrocytes (RBC) were used as representatives of normal tissues. All sera were inactivated at 56°C for 30 minutes to prevent possible lysis of mouse erythrocytes.

**Table 1.** Agglutination titers of the antisera and normal rabbit serum.

antiserum	antigen		
	Ehrlich carcinoma	R. B. C.	liver
anti-Ehrlich ascites carcinoma serum (AES)	5,120	80	40
anti-normal mouse tissue serum (ANS)	640	320	640
normal rabbit serum (NRS)	0	0	0

All sera were inactivated for 30 minutes at 56°C.

Of nine agglutination titers given in the table, that of anti-Ehrlich ascites carcinoma serum (AES) against EAC cells, 1:5,120, was conspicuously higher than the others. AES also agglutinated RBC and liver cells but only up to the final serum dilution of 1:80 and 1:40. Anti-normal mouse tissue serum (ANS) showed a much lower but still distinct agglutination titer against EAC. It also agglutinated RBC and liver cells to a similar extent. NRS did not agglutinate any of the test antigens even at the original concentration.

**Table 2.** Toxicity of the antisera in normal C3H mice.

antiserum dose	A E S						A N S			
	5.0ml	3.0	2.0	1.0	0.5	0.25	2.0	1.0	0.5	0.25
*	5/5	4/5	2/5	0/5	0/5	0/5	5/5	2/5	0/5	0/5

\* Denominator signifies the number of mice receiving intraperitoneal injection of the antisera; the numerator, the number of mice dying.

Table 2 shows the *in vivo* toxic effect of AES and ANS in normal C3H mice. Each group of five mice received intraperitoneal inoculation of from 0.25 to 5.0 ml per mouse of AES or ANS. Administration of larger doses of the antisera resulted in death of all or most of the animals within two to three days of marked hematuria. Loss of weight occurred in the groups given smaller doses of the antisera. Some animals of the groups died between 10 to 14 days of emaciation but others recovered weight and

survived. Administration of less than 1.0 ml of AES or 0.5ml of ANS did not apparently affect the normal mice and all survived without temporary weight loss. In most of the experiments presented later, AES and ANS were administered in a dose of 0.5 or 0.25 ml. Because such a dose seldom showed toxic side-effect, AES and ANS were routinely used without absorption with normal tissues. Absorbed antisera were employed only in such experiments as were designed to determine the specificity of the antisera.

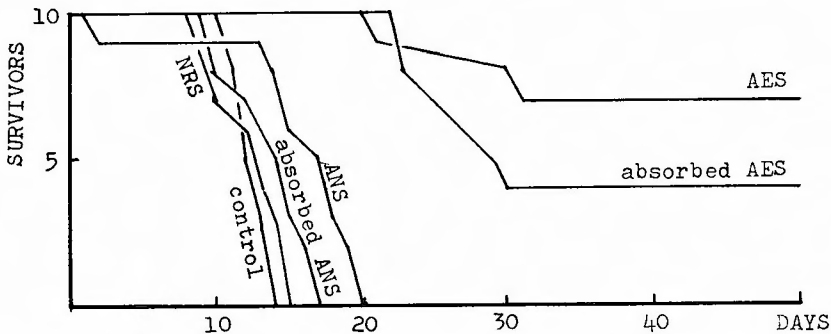


Fig. 1. 0.5 ml of each serum was intraperitoneally administered immediately after inoculation of 1 million Ehrlich ascites carcinoma cells.

Abbreviations: AES=anti-Ehrlich ascites carcinoma serum, ANS=anti-normal tissue serum, NRS=normal rabbit serum.

Fig. 1 illustrates the results of *in vivo* neutralization test of EAC with AES or ANS. In this experiment, 0.5ml of one of the antisera was administered immediately after inoculation of one million EAC cells to each mouse. Each group consisted of ten mice. Administration of a larger dose of the antisera at an earlier stage of the tumor growth than what was routinely employed in most of the experiments to be presented later, was preferred so that the difference in the tumor-inactivating capacities of the antisera will be more clearly demonstrated. Inactivated NRS showed no protection and all the mice succumbed to the tumor growth almost as rapidly as untreated controls. ANS slightly protected the mice against tumor growth and some life prolongation was observed in nine mice in which the tumor developed. However, one mouse died of hematuria, within 48 hours after injection of ANS in spite of the fact that 0.5ml of ANS is not toxic to a normal mouse. Perhaps tumor-bearing mice were less resistant against toxicity of ANS than normal ones. The strongest tumor inhibition was observed in the group injected with AES. Tumor developed in only three of ten mice and even those killed by the tumor growth showed a marked prolongation of mean survival time.

The difference in the tumor-inactivating capacities of AES and ANS could be a mere reflection of their quantitative difference in EAC agglutinating titers. Yet, the difference could also be qualitative: the protective power of ANS may be dependent on antibodies directed against antigens common to normal mouse tissues and EAC, while that of AES is specific to EAC. If such is the case, absorption with normal tissues should reduce the effect of ANS, but not AES. Both antisera were absorbed with "mixed normal cell suspension" and their *in vitro* agglutination titers and *in vivo* tumor-inactivating capacities were tested. The absorption reduced their agglutination titers against mouse erythrocytes

and liver cells to 1:10 or 1:20. Absorbed AES and ANS agglutinated EAC cells at the final serum dilution of 1:2,560 and 1:30, respectively. Absorbed ANS showed little tumor inhibition *in vivo*. As a matter of fact, all the mice treated with 0.5 ml of absorbed ANS succumbed to tumor growth and their mean survival time was 14.0 days, whereas that of the untreated control was 12.0 days. The absorption also diminished the *in vivo* tumor inhibiting effect of AES, but absorbed AES still gave a remarkable protection. Tumor developed in six mice out of ten and mean survival time of the dying mice was no shorter than that of the group treated with unabsorbed AES.

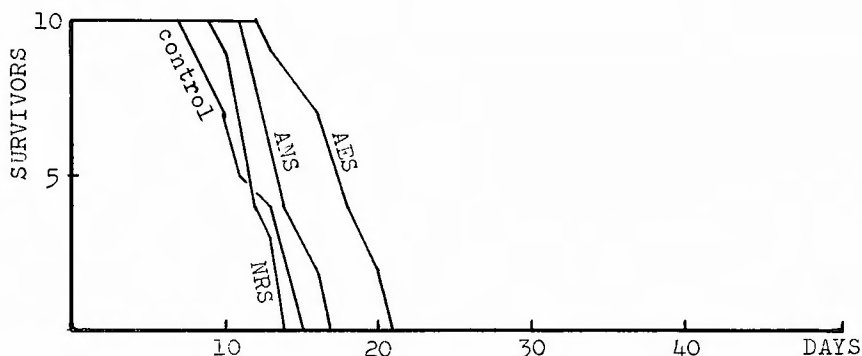


Fig. 2. 0.25 ml of each serum was intraperitoneally administered 48 hours after inoculation of one million Ehrlich ascites carcinoma cells. All abbreviations are same as in Fig. 1.

In the next experiment, AES, ANS or NRS were intraperitoneally administered in 0.25 ml dose 48 hours after inoculation of one million EAC cells.

The results are presented in Fig. 2. The mean survival time of each group and untreated control was 18.1, 14.6, 12.3 and 12.1 days, respectively. AES gave a slight protection at this stage of tumor growth. The prolongation of mean survival time caused by ANS or NRS injection, however, was no more than the possible statistical deviation. Thus, passive immunity induced by administration of 0.25 ml of the antisera could not cause the tumor to regress significantly after it had been inoculated for 48 hours.

In further studies of passive tumor immunity, it seemed desirable to determine if the

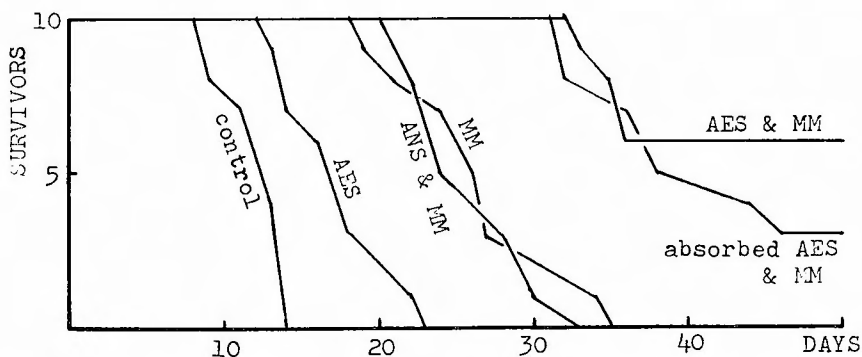


Fig. 3. 0.25 ml of each serum and/or 20 $\gamma$  of MM were administered intraperitoneally 48 hours after inoculation of 1 million Ehrlich ascites carcinoma cells. Abbreviations : MM=mitomycin. Other abbreviations are same as in Fig. 1.

induced immunity, subtle as it is, may favor the result of the cancer chemotherapy. Fig. 3 shows the result of cancer chemotherapy in mice immunized with AES. Intraperitoneal injection of  $20 \gamma$  of mitomycin (MM) resulted in the mean survival time of 27.3 days, or 14.9 days life prolongation over untreated controls, whose average survival time was 12.4 days. Because immunity induced by injection of AES 0.25 ml gave 5.0 days life prolongation, administration of MM  $20 \gamma$  and AES 0.25 ml will be expected to give  $14.9 + 5.0$  or 19.9 days life prolongation, if they affect the tumor growth independently. MM  $20 \gamma$  and AES 0.25 ml were injected to a group of ten mice 48 hours after inoculation of one million EAC cells. Tumor grew in four mice and killed them at an average of 36.0 days. Tumor did not develop in six during a 50-day period of observation. The result was by far better than what would be expected from merely integrating the individual effects of MM and the antiserum. Because 0.25 ml of AES is scarcely effective by itself to 48-hour old tumor growth, there must have been some unknown correlation between the immunity induced by the antiserum and the antineoplastic effect of MM, by which the over-all therapeutic result was conspicuously enhanced.

In order to determine if the immunity responsible for such enhancement is related to normal mouse tissue agglutinating antibodies, AES absorbed with "mixed normal cell suspension" was substituted. Absorbed AES and MM gave a slightly reduced therapeutic effect compared to unabsorbed AES and MM: seven mice out of ten died with a mean survival time of 37.9 days and three showed no tumor growth. On the contrary, MM  $20 \gamma$  and ANS 0.25 ml afforded no more protection than did MM  $20 \gamma$  alone. These two experiments indicated beyond reasonable doubt that normal mouse cell agglutinating antibodies were not concerned with the ones which augmented the effect of cancer chemotherapy.

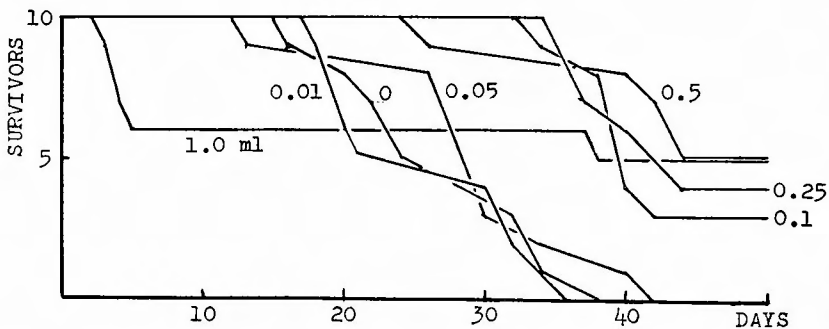


Fig. 4.  $20 \gamma$  of mitomycin was administered with varying doses of AES, ranging from 0 to 1.0 ml. Numbers by each line denote volumes in ml of AES given.

Fig. 4 shows the results of experiments designed to estimate minimal dose of AES that can induce immunity which would enhance the effect of MM.  $20 \gamma$  of MM was intraperitoneally injected with varying doses of AES, ranging from 0.01 to 1.0 ml, 48 hours after the inoculation of EAC. The therapeutic effects can clearly be separated into three different groups. The first group consisted of those treated with MM  $20 \gamma$  and 0.01 or 0.05 ml of AES. It is clear from the result that the simultaneous administration of less

than 0.05 ml of AES did not enhance the therapeutic effect of MM. Such treatment gave no more protection than did MM 20  $\gamma$  alone. Administration of 0.1, 0.25 or 0.5 ml of AES with MM 20  $\gamma$  resulted in a marked improvement of the therapeutic result. Number of survivors and mean survival time of dying mice being within a statistical deviation, immunity induced by any amount of AES within the given range of the dose enhanced the effect of MM to practically the same extent.

The results again suggested that AES and MM did not affect EAC independently but there existed some inherent correlation between their modes of action whose integrated process improved the over-all therapeutic result. 1.0 ml of AES and MM 20  $\gamma$  produced a striking toxic side-effect and killed four mice out of ten within 72 hours, though the rest of the mice, except one, did not show tumor growth. Because 1.0 ml of AES was not toxic to normal mouse, toxicity of MM and decrease in mouse resistance due to tumor growth may have to be accounted for.

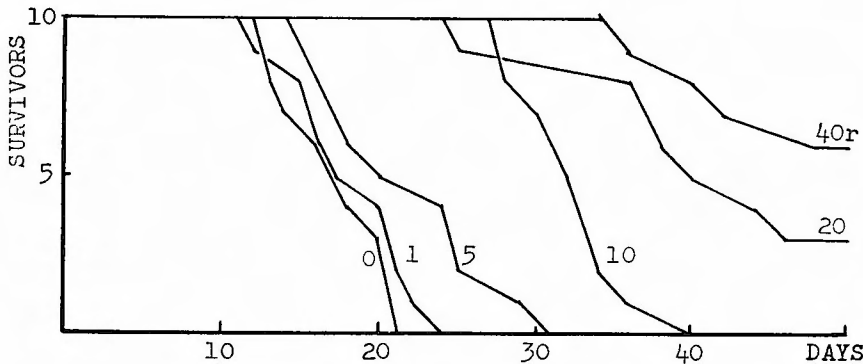


Fig. 5. 0.25 ml of AES administered with varying doses of mitomycin, ranging from 0 to 40  $\gamma$ . Numbers by each line denote amounts in  $\gamma$ 's of mitomycin.

Fig. 5 shows effects of various doses of MM in mice immunized with 0.25 ml of AES. Six groups of ten mice were injected with 0.25 ml of AES 48 hours after inoculation of one million EAC, and at the same time, 1 to 40  $\gamma$  per mouse of MM was administered. Increase in the amount of MM was simply paralleled with that in the mean survival time and number of negative tumor growths.

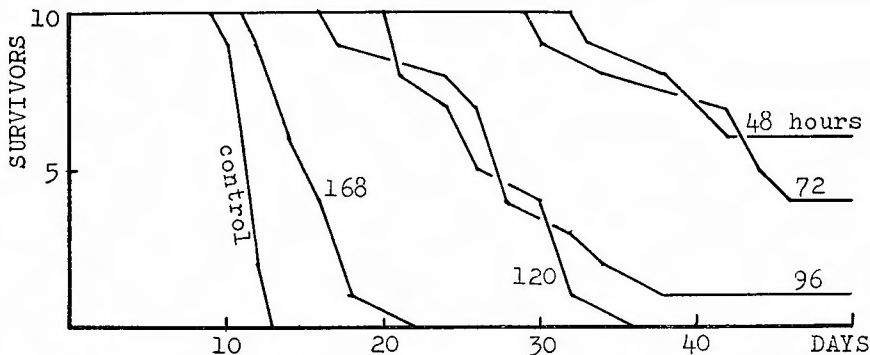


Fig. 6. 20  $\gamma$  of mitomycin and 0.25 ml of AES were administered at different space of time after tumor inoculation. Numbers by each line denote intervals in hours between the inoculation and the treatment.

Fig. 6 shows the results of experiments to determine the period of time that the initiation of treatment could be delayed, still preserving the inhibitory effect on the tumor growth. In these experiments, 0.25 ml of AES and MM 20 $\gamma$  were injected 48, 72, 96, 120 or 168 hours after inoculation of one million EAC. The data show that the treatment was just as effective when it was given at 72 hours as when given 48 hours after inoculation. However, when the injection was made at the 96th hour or later, the therapeutic effect rapidly decreased and the 168th hour (7th day) injection gave scarcely any protection compared to untreated control.

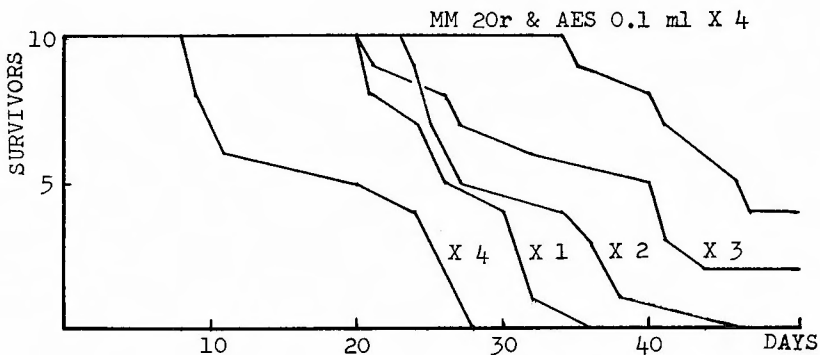


Fig. 7. Administration of 20 $\gamma$  of mitomycin and 0.25 ml of AES was repeated every 24 hours starting 120 hours after tumor inoculation. Numbers by each line denote numbers of repetition of the treatment.

The preceding experiments showed that 120-hour old tumor scarcely responded to the administration of AES 0.25 ml and MM 20 $\gamma$ . Success in experimental chemotherapy of well-established tumors have been reported, but it required too large a dose of chemotherapeutic agents to be applied to the management of human cancer. The experiments whose results are presented in Fig. 7 are designed to show if the injection is repeated, a relatively smaller dose of MM and AES may cause regression of 5-day old tumors. The treatment was started 120 hours after inoculation of one million EAC and repeated every 24 hours two to four times. Each dose was MM 20 $\gamma$  and AES 0.25 ml. When the injection was repeated two to three times at 24 hours interval, the mean survival time was prolonged and tumors regressed in two mice out of ten that received three injections. On the other hand, mice receiving four successive injections died of hematuria and/or emaciation. The response was similar to what was observed with normal mice which received an excessive amount of AES. It has already been shown that 0.1ml of AES enhanced the effect of MM as much as did 0.25 ml of AES. Therefore, 0.1ml of AES and MM 20 $\gamma$  were administered four times at 24 hour intervals. The treatment gave an excellent result with marked life prolongation and regression of the tumors in 40% of the mice.

Figs. 8, 9 and 10 show that a similar effect can be seen with some other anti-cancer chemotherapeutic agents administered with AES. Under the given experimental conditions, toyomycin and carcinophilline gave the most favorable results; endoxane and nitromine followed. The effect of merphyrine and trespamine, however, were not significantly enhanced by AES.



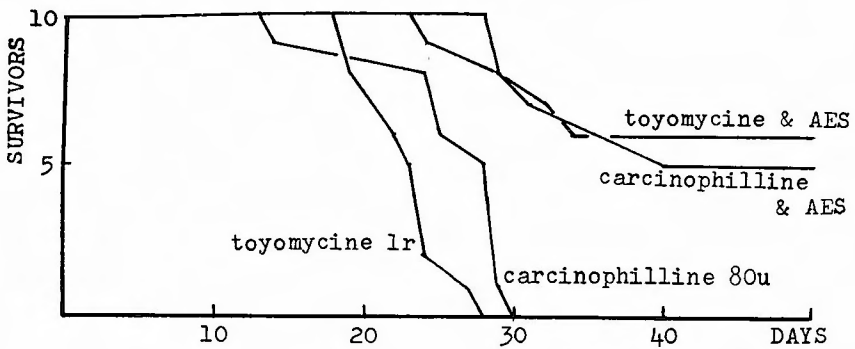


Fig. 8. Toyomycine 1r or carcinophilline 80u was administered alone or with 0.25 ml of AES.

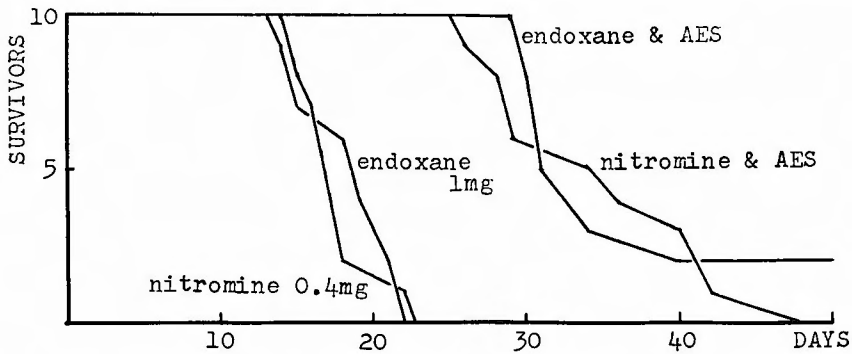


Fig. 9. Nitromine 0.4 mg or endoxane 1 mg was administered alone or with 0.25 ml of AES.

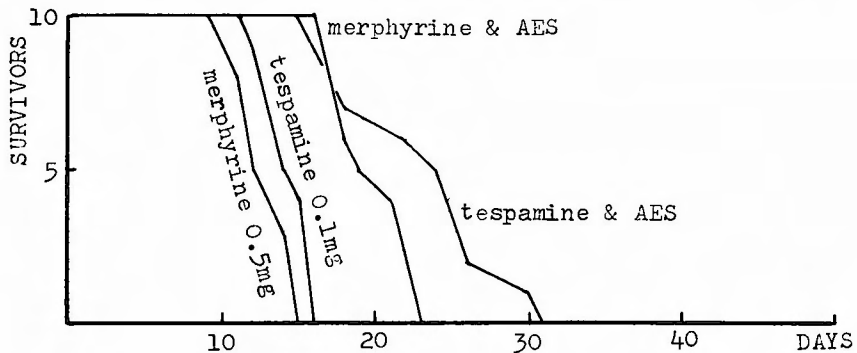


Fig. 10. Merphyrine 0.5mg or tespamine 0.1mg was administered alone or with 0.25 ml AES.

### DISCUSSIONS

The question of tumor immunity and its specificity is a subject long disputed, but with little fruitful results. Exhaustive reviews of this field have been made by many authors.<sup>4) 27) 50) 62) 63) 64) 67)</sup>

In this paper, the term "immunity" will be used only to denote the property of being able to produce resistance to the tumor growth, without any implication concerning the

biologic nature of the process.

Tumor immunity falls into two categories, namely active and passive immunity.

Numerous reports which claimed success in active immunization against transplantable animal tumors have been proved erroneous since introduction of inbred animals to the study of cancer and subsequent development of immunogenetics in tumor transplantation.<sup>3) 9) 11) 18) 30) 37) 80) 61)</sup> Neoplasms originated in random-bred animals, or those which arose from an inbred strain and propagated in genetically different strains, are to a large extent foreign to the host and, therefore, bring about foreign protein reactions in the engrafted hosts. The reactions are identical with the immunity which is the unwanted but usual result of homografting or heterografting normal tissues: they are not directed against the tumor *per se* but rather against antigens common to the tumor and normal tissues of the animal in which the tumor originated.<sup>55)</sup> Nevertheless, there are a few cases in which animals of seemingly inbred strains were immunized against autochthonous tumors.<sup>19) 40) 41) 42) 46) 47) 57) 65)</sup> These were attributed to possible genetic deviations between host and tumor which developed by mutation during long period of serial transfer<sup>27)</sup> or to residual heterogeneity which any inbred strains inevitably contain.<sup>55)</sup>

There are also reports that methylcholanthrene-induced tumors are antigenic in their isologous hosts<sup>17) 24) 25) 39) 53) 58)</sup> and even in the host of origin.<sup>38)</sup> A similar result was reported with a tumor induced by another aromatic hydrocarbon, dibenz(a, h)anthracene.<sup>56)</sup> Carcinogen induced-tumors possess particular antigens which do not exist in spontaneous tumors.<sup>55) 58)</sup> Therefore, observations in induced-tumor experimentations cannot be applied to human cancer. Any studies in tumor vaccination have no carry-over to the problem of human cancer unless the studies were made with recently arisen spontaneous tumors of highly inbred strains transplanted to members of the same subline of the same strain in which they first appeared.<sup>37)</sup> Sometimes it is necessary to estimate residual heterogeneity among members of a subline by exchange skin grafting,<sup>55)</sup> which is regarded as a test for at least fifteen histocompatibility loci.<sup>2)</sup> Experiments performed under such strict immunogenetic considerations showed<sup>13) 16) 17) 29) 55) 58)</sup> that routine vaccination techniques, which would inhibit the growth of induced or long-transplanted tumors, were ineffective in a genetically identical tumor-host system.

Heterografting tumor tissues, whether alive or dead, provoke immune reactions in engrafted animals and usually result in the appearance of circulating antibodies in the peripheral blood. It has been repeatedly demonstrated that antitumor sera thus prepared in heterologous hosts will inhibit growth of that tumor *in vitro*<sup>7) 10) 12) 33) 35) 43) 44) 51) 70)</sup> or if administered prophylactically to an animal just before implantation of the same type of tumor.<sup>6) 22) 23) 32) 45) 49) 70)</sup> Therapeutic results were also reported in some animal experiments, when a larger dose of heteroimmune serum was administered within 48 hours after transplantation.<sup>1) 5) 15) 31) 52) 54) 59) 69)</sup> On the other hand, animals with well-established tumors responded to the administration of heteroimmune sera with only temporary regression of the tumors.<sup>5)</sup>

Results of the prophylactic administration of AES in the present experiment are in good agreement with the reports cited above. Serotherapy of 48-hour old tumor, however,

resulted in but a slight prolongation of mean survival time, though some authors reported regression of 48-hour old tumor by antiserum injection.<sup>1) 5) 52) 54)</sup> The difference can be explained on the basis of antiserum dose. In the present experiment, AES was administered in a dose of 0.25 ml, whereas tumor regression was reported only when more than 1.0 ml of antiserum was injected.

Effect of tumor serotherapy decays very rapidly as the initiation of the therapy is delayed. Serotherapy is almost ineffective once the tumor has established itself in the engrafted host. BUINAUSKAS, using 1,000 mg/kg of  $\gamma$ -globuline isolated from anti-Walker tumor serum which was prepared in sheep, treated Walker tumor of 10 mm in diameter growing in rats.<sup>5)</sup> The result was just a temporary shrinkage of the tumor, and it started growing again after a few days.

Inability of the heteroimmune sera to produce a permanent regression of a well-established tumor is due, to a large extent, to quantitative deficiency of antibodies administered *versus* tumor cells to be affected. Multiplication of tumor cells, as is typically seen with EAC, is quite speedy. The total cell count may increase sixty-fold in 120 hours.<sup>36)</sup> McALLISTER<sup>48)</sup> showed that the amount of antiserum necessary to inactivate HeLa cells in conditioned rats increased concomitantly with the number of tumor cells. If this be also true with EAC in mice, (as many experiments indicated that at least 1 ml of antiserum is necessary to inactivate one million EAC cells *in vivo*) simple algebra will show that more than 60 ml of antiserum is necessary to inactivate the EAC cells 120 hours following inoculation of one million cells. This indicates a practical limit to the use of antisera in the treatment of advanced cancer. As a matter of fact, the amount of antiserum practically administrable to an experimental animal or a cancer patient without intolerable side-effect is far less than what would be necessary to destroy a large tumor mass. Thus, cancer serotherapy is effective only as a prophylactic measure.

The most important problem in tumor serotherapy is whether the effect of the antiserum is directed specifically to the tumor. In active immunization, the problem is simple. In a highly isologous tumor-host system, any normal antigens which exist in the tumor are not foreign to the host, and, therefore, cannot exert antibody production in the host. If any immune reactions would be observed in such a system, they can be regarded as a proof that there exists an antigen or antigens unique to the tumor. Observations so far reported claim that no immune reactions are detectable in a highly isologous tumor-host system. Two hypotheses were proposed in explaining the failure of spontaneous tumors to produce immunity in isologous hosts. One is that there is no antigenic difference between tumor and host and, therefore, induction of either active or passive tumor immunity is theoretically impossible. The other is that there may be antigens unique to the tumor, but they are not foreign to the original or isologous hosts and are not antigenic to them. The latter hypothesis suggests the possibility that such antigens could be antigenic in heterologous animals. However, both are unsubstantiated speculations.

In passive immunization, antibodies are produced in a species of animal different from that in which the tumor first arose. Foreignness of antigens is virtually out of the question: all macromolecular constituents of tumor cells are now potentially antigenic, regardless of their capacity to provoke an immune response in the host of origin. Accepting the assump-

tion that there are antigens unique to the tumor and that they can produce antibodies in heterologous animals, the problem is to distinguish the antibodies directed to normal cell components from those directed specifically to the tumor. EAC mostly consists of tumor cells, but is still contaminated with a trace of RBC and WBC. Even within a single EAC cell, there are, besides possible cancer specific antigens, a large proportion of antigens which are shared in common with normal cells. Therefore, even an antiserum produced against EAC could contain antibodies to normal tissues as well as the desired anticancer antibodies. Such antibodies to normal tissues will also agglutinate the tumor cells *in vitro* or neutralize them *in vivo* by affecting the normal components of the tumor cells rather than antigens unique to the tumor. As a matter of fact, ANS which was produced against normal mouse tissues, affected EAC both *in vitro* and *in vivo*, though the cytotoxic effect was much smaller than that of AES. Therefore, neither agglutinating nor neutralizing test can distinguish tumor specific antibodies from anti-normal antibodies.

Experiments were performed to determine what kind of antibodies compose a major factor in the antineoplastic effect of AES. AES was absorbed with normal cells which would include antigens corresponding to the antinormal antibodies in the antiserum. If anti-normal antibodies are the major factor in the antineoplastic effect of AES, the absorbed AES will no longer neutralize the tumor cells. As described by WITEBSKY,<sup>71)</sup> antigens in cancer tissue are species, organ or cancer specific. In order to remove organ and species specific antibodies, AES was absorbed with mammary gland tissue (EAC first originated in mammary gland) and with representative tissues of the same species of animal: namely liver, kidney and erythrocytes of C3H mice. The absorption removed from AES most of the antibodies which agglutinate mouse erythrocyte and liver cell. EAC agglutinating antibodies were only partially removed by the absorption. Absorbed AES neutralized EAC *in vivo* almost as strongly as did unabsorbed AES. This is in agreement with the results of absorption of anti-cancer serum with fatty tissue or erythrocytes.<sup>53) 8) 54)</sup> It was reported that the absorption did not significantly reduce the effect of the antiserum against tumor growth. On the other hand, absorption of ANS with the same antigens resulted in the marked reduction of tumor agglutinating and neutralizing capacities of the antiserum. These two antagonistic phenomena can be explained by assuming that the active agents in AES and ANS are different. Most of the antibodies in AES are specific to EAC and, therefore, cannot be absorbed with normal cells, whereas antibodies in ANS are anti-normal antibodies subject to absorption with normal cells. An objection could be made against the assumption. That is: antibodies to be absorbed are accessible only to the cell membrane, and the absorption method is inadequate to remove all anti-normal antibodies in AES, which was produced against whole cells. If such is the case, the same absorption should also fail for the same reason to remove anti-normal antibodies from ANS. As it was otherwise proved, surface antigens should be enough and adequate for removal of anti-normal antibodies.

Yet, the effect of absorbed AES cannot be wholly attributed to the effect of cancer specific antibodies. There may be a considerable difference in the amount of anti-normal antibodies in AES and that of corresponding antigens in the normal cells used for the absorption. Therefore, the "absorbed" AES may still contain not a small proportion of

unabsorbed anti-normal antibodies.

Administration of AES resulted in only a limited success in controlling tumor growth. The immunity it induced was so subtle that it even failed to inactivate 48-hour old tumor *in vivo*. The immunity, however, was proved to assume a great importance by collaborating with tumor chemotherapy. Effect of MM against EAC was dramatically enhanced in animals immunized with AES. Combined effect of MM and AES was by far the larger than what would be expected by simply summing up the individual effects of the two agents. If the two agents affected EAC independently, the effect of the combination therapy must have been much smaller. Assuming that MM is potentially as effective against cancer *in vivo* as it is *in vitro* but the efficacy is not manifested because of the lack of host immune reactions against cancer, the enhancement of the cancer chemotherapeutic effect will be more easily explained.

The prospect that the effect of cancer chemotherapy will be augmented if the cancer bearing host could be properly immunized has been postulated by some authors. KLEIN<sup>36)</sup> has claimed that one of the main difficulties that confront tumor chemotherapy, as contrasted to the chemotherapy of bacterial infections, lies in the fact that host resistance is of minor or no importance, and therapy has to aim at the destruction of tumor cells, without receiving major help from the defense mechanisms of the organism itself. SOUTHAM has postulated<sup>63)</sup> that, "The difference between infectious and neoplastic diseases in responsiveness to chemotherapy might well be due to the inadequacy of host defenses in the latter. If this be true, even a slight improvement in host-immune mechanisms might contribute significantly to the efficacy of chemotherapy. This would seem to be a type of combination therapy which merits further study." The present experiment realized the postulates presented by the two authors in, that immunized animals responded to cancer chemotherapy much better than non-immunized animals.

Tumor chemotherapy in animals with induced host resistance will be termed "immunochemotherapy." The immunochemotherapy can be divided into active and passive, depending upon whether the host resistance is induced actively or passively.

Chemotherapy of cancer in animals which had received "cancer vaccination," or active immunochemotherapy, was recently reported by ISHIBASHI et al.,<sup>34)</sup> and they claimed that the effect of the chemotherapy was impressively promoted by intradermal "immunization." Because the experiment was carried out along a non-isologous tumor-host system, the result must have been largely influenced by homograft reactions. Of the two tumors used in their study, one was Yoshida ascites sarcoma, which is a non-specific tumor, while the other was MH 134 ascites hepatoma, which is an induced and long-transplanted tumor: both are histoincompatible in any engrafted hosts. Based on their animal experiments, Ishibashi and his co-workers gave intradermal "autovaccination" to inoperable cancer patients, and claimed that immunological responses thus evoked favored the effect of cancer chemotherapy. However, the intradermal vaccination of experimental tumors which was seemingly effective in non-isologous tumor-host systems, cannot be applied to human cancer, because a human cancer is necessarily autochthonous to the patient and is free from the homograft reactions that blurred the interpretation of the result of the

animal experiment. MOORMAN<sup>63)</sup> also gave autovaccination to inoperable cancer patients in conjunction with chemotherapy. The evaluation of his work is afflicted with lack of animal experimentations substantiating the possibility of "cancer vaccination."

Cancer chemotherapy in passively immunized animals, or passive immunochemotherapy, has been reported by several workers. The first of these studies was reported by YOSHIO<sup>72)</sup> in 1951. Isoantiserum obtained from a rat which had a spontaneous regression of Yoshida ascites sarcoma was employed to induce immunity in rats. 48 hours after inoculation of Yoshida ascites sarcoma, antineoplastic chemicals such as colchicine, nitrogen mustard, urethan or "Na" were administered to the rats with or without combination of the isoantiserum. The effect of the anti-neoplastic chemicals was remarkably elevated by simultaneous administration of the isoantiserum. TAKEDA<sup>68)</sup> reported that passive immunity, induced by anti-Yoshida ascites sarcoma rabbit serum, enhanced the effect of nitromine, carcinophilline or RC4 against the sarcoma. A similar result was reported by the same author with Takeda sarcoma.

The present experiment falls into the category of passive immunochemotherapy and it revealed several facts concerning the mechanism of the therapy.

Participation of normal cell agglutinating antibodies seems to be of less importance in immunochemotherapy than in serotherapy. 0.25 ml of ANS induced a slight inhibition on 48-hour old tumor, but the same dose of ANS and MM 20  $\gamma$  gave no more protection than did MM 20  $\gamma$  alone. Besides, absorbed AES enhanced the effect of MM almost as strongly as did unabsorbed AES. Serotherapy of cancer is obviously favored by normal cell agglutinating antibodies which unwantedly but inevitably accompany heterologous anti-cancer serum. On the other hand, immunochemotherapy is scarcely influenced by their presence and is mostly dependent on the antibodies which cannot be absorbed with normal mouse tissues.

One of the most important facts revealed in the present experiments is that there is an all-or-none law between doses of AES and the over-all therapeutic results which the antiserum affords in collaboration with MM. Any dose of AES below a critical point did not enhance the effect of MM at all, while any doses above that always gave a maximal enhancement. The fact suggests that a simultaneous administration of AES and MM is not a simple combination therapy, but AES substitutes for the host defence mechanism which is lacking in cancer bearing host. Such host defence will participate with MM in the destruction of cancer cells, as is seen with chemotherapy of bacterial diseases. In other words, AES behaves as a catalyzer to MM in the sense that AES is almost ineffective by itself against cancer, but stimulates the effect of MM when given together.

Another important fact is that immunochemotherapy can cause regression of well-established tumors, which has never been achieved by simple serotherapy, and only incompletely with a larger dose of cancer chemotherapeutic agents. Four out of ten mice carrying 5-day old EAC were cured with four daily injections of MM 20  $\gamma$  and AES 0.1 ml. A total dose of AES, 0.4 ml, is entirely ineffective at this stage of tumor growth, when the estimated number of tumor cells exceeds 60 million.<sup>39)</sup> SUGIURA<sup>66)</sup> reported that 280  $\gamma$  of MM was necessary to destroy 7-day old EAC. Complete destruction of the tumor was observed two weeks after the treatment, but no cases of cure were reported after

longer period of observation. In the present experiment, immunity induced by an amount of antisera, which by itself is practically ineffective, promoted the effect of MM to such a degree that only 80% of MM caused complete regression of 5-day old well-established tumors in four mice out of ten. This may suggest a more practical method of administering antineoplastic agents to cancer patients with massive metastasis. As reported by several workers, fairly good amount of heterologous anticancer serum can be administered to cancer patients without clinically important side effects.<sup>5) 20) 53)</sup> The immune state thus produced in patients is not strong enough to destroy a large mass of cancer cells, but may enhance the effect of subsequent cancer chemotherapy, as was observed in animal experiments, and make the prognosis more favorable.

Immunity induced by AES can also enhance the effect of other cancer chemotherapeutic agents. Among six agents tested, two antibiotics, toyomycin and carcinophillin, were of best choice. Because MM is also an antibiotic, it was the impression that antibiotics were the drugs of choice in immunochemotherapy. Two alkylating agents, endoxane and nitromine, were also good partners of AES but their effects were not so much enhanced as those of the three antibiotics. Merphyrine and trespamine were proved to be ineffective against EAC when administered alone as well as in immunochemotherapy.

The present study is still incomplete as an experimental basis for clinical immunochemotherapy, because all the experiments were performed with EAC which is not completely histocompatible in C3H mice and is, therefore, under the influence of host defense mechanism. It is now beyond dispute that any studies in active immunization which are made without ample considerations concerning tumor-host relationship are meaningless. In passive immunization, however, genetic identity between tumor and host is not of prime importance as it is in active immunization, because the effect of histoincompatibility reaction can easily be overwhelmed by heteroantibodies administered and will not largely change outcome of the experiment. For the sake of scientific accuracy, however, it is desirable that a similar experiment be performed in a completely isologous tumor-host system. Besides, an ascitic form of carcinoma may have a larger sensitivity to serotherapy, because antibodies are more easily accessible to ascites tumor cells than to solid ones. Validity of immunochemotherapy has to be proved in solid and isologous tumors before it can be reasonably applied to human cancers.

## SUMMARY

1) Heterologous antisera were produced in rabbits against Ehrlich ascites carcinoma and normal C3H mouse tissues.

2) Anti-Ehrlich ascites carcinoma serum (AES) enhanced the cancer chemotherapeutic effect of mitomycin *in vivo*. Such enhancing effect was not abolished by absorption of the antiserum with normal mouse tissues.

3) Anti-normal mouse tissue serum did not show such enhancing effect.

4) The enhancing effect was not caused by antibodies directed to normal mouse antigens, but rather by antibodies unique to the tumor.

5) A term "immunochemotherapy" was proposed to denote chemotherapy of cancer

in animals immunized with anticancer immune serum.

6) The maximal enhancing effect was obtained by administration of 0.1 to 0.5ml of AES. Less than 0.05ml of AES was ineffective. All-or-none law was observed between AES dose and the enhancement.

7) Delay of initiation of the treatment resulted in a rapid decay of the therapeutic result.

8) When repeated, immunochemotherapy could cause regression of 5-day old well-established tumors.

9) AES similarly enhanced the effect of toyomycine, carcinophilline, endoxane and nitromine, but not that of trespamine and merphyrine.

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## 和文抄録

## 癌の免疫化学療法

担エールリッヒ腹水癌マウスに於ける抗癌剤の効果に  
及ぼす受動免疫の影響

京都大学医学部外科学教室第1講座（指導：荒木千里教授）

小 野 博 通

細菌性疾患に対する化学療法に比して癌の化学療法  
の効果が著しく劣ることは、抗癌剤自体の問題もさ  
ることながら、細菌感染に対して発揮されるが如き生  
体防衛機構が癌に対しては殆んど欠除していることが  
重大な原因の一つである。

エールリッヒ腹水癌（エ癌）を家兎に注射して抗血  
清を作成し、この抗血清をC3Hマウスに腹腔内投与す  
るとエ癌の発育を或る程度抑制することが出来るが、  
移植48時間以後のエ癌を消退させることは出来ず、担  
癌マウスの生存日数を僅かに延長し得るのみである。

しかし受動免疫を行なつた担癌マウスは抗癌剤によ

く反応し通常量よりはるかに少量の抗癌剤によりエ癌  
を消退或いは治癒させることが出来る。これは受動免  
疫により生体防衛機構の欠除がある程度代償され、抗  
癌剤の効果が増強されるためであると考えられる。

受動免疫下における癌の化学療法の効果は免疫と抗  
癌剤の単なる相加作用から期待されるよりはるかに著  
しいものがあり、かかる癌の治療方法は免疫化学療法  
と呼ぶのが適当と思われる。

免疫化学療法の機序、関与抗体の癌特異性、使用抗  
癌剤の差による効果の相違及び人癌に対する応用の可  
能性が検討された。