EXPERIMENTAL ANTICANCER STUDIES

Part 28. Effect of Living Hemolytic Streptococci on Various Transplantable Ascites Tumor Cells in *In Vitro-In Vivo* Assay System*

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It was in 1954-55, when living hemolytic streptococci were shown to have a characteristic property to inhibit the invasion power of Ehrlich ascites carcinoma cells in mice[1]. In this preliminary anticancer experiment, the so-called ***in vitro-in vivo*** assay method was employed.

Since then, a number of experiments[2)-14)] relating to the anticancer activity of living hemolytic streptococci have been performed in our laboratories**.

During past 8 years, there appeared many papers reporting the anticancer activity of hemolytic streptococci from the laboratories in abroad[16)-23)].

The animal tumors, which have so far been used in the anticancer experiments with hemolytic streptococci, are five mouse tumors, Ehrlich carcinoma,[1)-4),17),18),22),23)] Krebs-2 carcinoma[19)], Sarcoma 37[19)], Leukemia 1210[22)], lymphatic leukemia L 4946[16)], one rat tumor, fibrosarcoma induced by 3:4-benzpyrene[24)], and one rabbit tumor, Brown-Pearce carcinoma[20),21)]. It is desirable to further investigate whether hemolytic streptococci are capable of eliciting anticancer activity against any malignant tumor cells other than those described above.

The present experiments with six different kinds of transplantable animal tumors

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* The results of this work were presented partly at the 18th General Meeting of the Japanese Cancer Association, Tokyo, 1959.

** Quite recently, significant advance in the anticancer studies has been made in our laboratories by the finding that the preparation of *in vitro* penicillin-pretreated hemolytic streptococci was at least 20 times more effective than non-pretreated streptococci[15)].

were undertaken in 1958 for the purpose of obtaining further information on the problem of the antagonism between hemolytic streptococci and tumors.

In addition, the results of experiments, in which the production of streptolysin S in the incubation mixtures of living hemolytic streptococci and tumor cells was examined, will also be presented.

MATERIALS AND METHODS

1. Animals:
   a) Male albino mice of dd strain, weighing 17-20 gm were used for the experiments with mouse ascites tumors.
   b) Male hybrid rats were used for the experiments with rat ascites tumors.
2. Strain of *Streptococcus hemolyticus*:
   A stock laboratory strain of Group A streptococcus "Su" was used throughout.
3. Phosphate-Ringer's solution, pH 7.2 (P B R):
   This bufferized Ringer's solution consists of one part of M/15 phosphate buffer (pH 7.2) and four parts of Mammalian Ringer's solution.
4. Penicillin solution:
   A solution of crystalline potassium penicillin G in normal saline, 10×10^4 units per ml, was prepared. Penicillin was used for protecting experimental animals from streptococcal infection.
5. Streptococcal suspension:
   0.2 ml of 20-hour culture of hemolytic streptococci was seeded into 200 ml of ordinary broth (pH 7.3) and incubated at 37°C for 20 hours. The cocci were harvested by centrifugation, and the sedimented cocci, after being washed twice with chilled PBR (50 ml for each), were resuspended in 10 ml of PBR, giving a 20-fold streptococcal concentration of the original culture volume.
   A part of this streptococcal suspension was heated at 100°C for 30 minutes (heat-killed streptococci suspension).
6. Tumor cell suspension:
   Three types of mouse ascites tumor, Ehrlich carcinoma, Sarcoma 180, and Leukemia SN 36, and two types of rat ascites tumor, Yoshida sarcoma, and ascites hepatomas AH 130 and AH 66, were employed in the present experiments.
   The procedures for preparing the tumor cell suspensions are as follows:
   a) In cases of experiments with mouse tumors, ascitic fluid was withdrawn from donors bearing 7-day-old ascites tumors, and centrifuged. The tumor cells thus collected, after being washed twice with chilled PBR, were resuspended in PBR to contain 80 million tumor cells per ml.
   b) In cases of experiments with rat tumors, ascites tumor cells were harvested from donors bearing 5 or 6-day-old tumors. The tumor cells, after being washed twice with chilled PBR, were resuspended in appropriate volume of PBR, number of tumor cells being 50 million per ml for Yoshida sarcoma cell suspension, and 25 million per ml for suspension of both ascites hepatoma cells.

7. Anticancer experiments:

The anticancer experiments were performed according to in *in vitro-in vivo* technique as described in the previous paper[1].

a) Experiment with a suspending mixture of hemolytic streptococci and Ehrlich carcinoma cells in relation to its incubation temperature.—— Four ml of the carcinoma cell suspension was mixed with 8 ml of the streptococcal suspension and 4 ml of PBR, and subsequently the suspending mixture was divided into four parts of equal volume. Each of 4 ml aliquot was then allowed to stand at 0°C, 15°C, 27°C and 37°C, respectively. After 90 minutes, 1 ml of penicillin solution was added to each of the mixtures thus treated, and 0.5 ml of this suspending mixture was inoculated intraperitoneally to each of the corresponding group of animals.

b) Experiments on the effect of living hemolytic streptococci against different types of ascites tumor.—— A mixture of 2 ml of a tumor cell suspension, 4 ml of living (or heat-killed) streptococcal suspension and 2 ml of PBR was incubated at 37°C for 90 minutes. At the end of the incubation period, to 6 ml withdrawn from the mixture was added 1.5 ml of penicillin solution, and 0.5 ml of this suspending mixture was inoculated intraperitoneally to each animal.

The remaining part (2 ml) of the incubated mixture was used for determining streptolysin S produced in the mixture.

For control purpose, a mixture of 2 ml of tumor cell suspension and 6 ml of PBR was prepared, and incubated at 37°C for 90 minutes. At the end of the incubation period, 1.5 ml of penicillin solution was added to 6 ml of the control mixture, and 0.5 ml of this suspending mixture was inoculated intraperitoneally to each control animal. The remaining 2 ml was used for streptolysin S determination.

On the first 3 days following the inoculation, a daily dose of 10,000 units of penicillin was given to all experimental animals.

c) Determination of results.—— Animals which died during the experimental period were examined for the cause of death, and animals still alived 50 days after the inoculation were sacrificed and autopsied. Number of survivors and existence of tumor growth on the terminal of the experiments were used as indices for criterion of antitumor effect.

8. Hemolysis test:

The remaining 2 ml of the incubated mixtures above-mentioned was centrifuged. The clear supernatant fluid thus obtained was diluted with normal saline serially. To 1 ml of each diluted supernatant, 1 ml of 1 % washed rabbit erythrocyte suspension was added, and placed in a 37°C-water bath for 2 hours.

RESULTS AND DISCUSSIONS

I. Anticancer effect shown by a suspending mixture of hemolytic streptococci and Ehrlich carcinoma cells in relation to its incubation temperature

Prior to going into the present work, a series of *in vitro-in vivo* anticancer ex-

periments were performed in order to define optimal temperature for incubating a suspending mixture of living hemolytic streptococci and tumor cells. The results obtained in this kind of experiments are presented in Table 1. As can be seen from this table, anticancer effect of living streptococci could be fully expected, only when a mixture of the tumor cell suspension and the streptococcal suspension was placed at 37°C for 90 minutes; all 5 animals lived even after 50 days without macroscopical tumor findings, while all animals received a suspending mixture, which had been placed for 90 minutes at temperature lower than 15°C, succumbed to the tumor growth within 40 days.

All control animals inoculated with tumor cells alone, regardless of the temperature at which they were exposed died of tumor invasion within 30 days.

On the basis of these results, following experiments for testing anticancer activity of living hemolytic streptococci on five different types of transplantable animal tumors were carried out by adopting an incubation condition of the suspending mixture at temperature of 37°C.

II. A. Effect on mouse ascites tumors

Tables 2,3 and 4 show the results of the anticancer experiments, in which antitumor effect of living hemolytic streptococci on three different types of mouse ascites tumor, Ehrlich carcinoma, Sarcoma 180 and lymphatic leukemia SN 36, was examined.

The significant results in this series of experiments are as follows:

1) In the experiments upon Ehrlich carcinoma, an definite antitumor effect of hemolytic streptococci was confirmed, as it has been observed by Koshimura, *et al.* in their earlier experiments.[1),5)] (Tables 1 and 2).
2) Living hemolytic streptococci were also effective in inhibiting the invasiveness of Sarcoma 180 cells in mice; 9 out of 10 mice inoculated with sarcoma cells plus living streptococci lived quite healthy even 50 days after the inoculation, when all the animals were autopsied without macroscopical tumor findings (Table 3).
3) The streptococci were also effectively tested upon Leukemia SN 36; out of 10 mice received a mixture of the tumor cells and living streptococci 5 lived after 50 days with no macroscopical finding of tumor invasion, while remaining 5 animals died of tumor growth within 36 days (Table 4).
4) On the contrary, in every cases of experiments performed with heat-killed (100°C, 30 min.) streptococci all animals died of invasion of tumor cells (Ehrlich carcinoma, Sarcoma 180 and Leukemia SN 36) as rapidly as the control animals received tumor cells alone.

B. Effect on rat ascites tumors

In regard to the problem of the anticancer activity of hemolytic streptococci, two different types of rat ascites tumor were included in this series of experiments. The results of the experiments are presented in Tables 5, 6 and 7. As may be seen from these tables, it was also demonstrated that living hemolytic streptococci possessed of a potent antitumor activity against Yoshida sarcoma, and ascites hepatomas AH 130 and

AH 66; survival time of the animals inoculated intraperitoneally with a mixture of Yoshida sarcoma cells and living streptococci was substantially increased, and 4 out of 10 test animals lived in quite good health even after 50 days of the inoculation. Survivors with no tumor finding on the terminal of the experiment were 4 out of 10 rats received a mixture of AH 130 cells and living streptococci, and 7 out of 10 rats received a mixture of AH 66 cells and living streptococci.

However, animals inoculated with a mixture of heat-killed streptococci and either of the above-mentioned rat tumor cells all died of tumor invasion within 20 days, as was in the case of control animals.

Thus, it was demonstrated that all six different transplantable animal tumors, Ehrlich carcinoma, Sarcoma 180, Leukemia SN 36, Yoshida sarcoma, and ascites hepatomas AH 130 and AH 66 involved in the present experiments, were sensitive to hemolytic streptococci. However, the figures in Tables 2-7 are not to be directly compared as to the difference in the sensitivity of these six different tumors to the streptococci.

Further, in view of point of the anticancer problem, it seems to be worthy to mention here that hemolytic streptococci was found to be effective in inhibiting the invasion power of AH 66 cells, which is known to be insensitive[25] to nitromin.

III. Production of streptolysin S in the incubated mixture of living hemolytic streptococci and tumor cells

It has been demonstrated in our laboratories[26] that when a suspension of washed living hemolytic streptococci and washed tumor cells (Ehrlich ascites carcinoma cells as well as Yoshida sarcoma cells) was incubated at 37°C, appreciable amounts of streptolysin S was produced in the medium within a short time.

At first, the production of streptolysin S in the medium was simply thought to be a sign of interaction of streptococci and nucleic acid contained in the tumor cells[2)-4)].

In view of this finding, it was of interest to ascertain whether the streptolysin S production may occur in the incubation mixtures of living streptococci and tumor cells used in the above-mentioned experiments. Therefore, prior to the addition of penicillin, an aliquot of sample was withdrawn from each of the mixtures after 1.5 hour-incubation at 37°C, and centrifuged. The clear supernatant thus obtained were subjected to the estimation of hemolytic power. The results are presented in Table 8.

At a glance, it may be seen from the table that the clear supernatants from the incubation mixtures of living streptococci and tumor cells, regardless of their different types, were found to be hemolytic up to a dilution of 1 in 32 (~16).

The heat-killed streptococci, however, were found to have entirely lost their ability to produce the hemolysin in the presence of tumor cells, i.e. none of the supernatants in these series (II') of experiments caused hemolysis even in a dilution of 1:2.

It is significant that despite of the differences in kinds of tumor cells exposed to living hemolytic streptococci, about equal amounts of streptolysin S were produced in all suspending mixtures.

SUMMARY

Employing *in vitro-in vivo* assay system, anticancer activity of living hemolytic streptococci upon six diferrent kinds of transplantable ascites animal tumor cells was examined. These included three mouse tumors, Ehrlich carcinoma, Sarcoma 180, Leukemia SN 36, and three rat tumors, Yoshida sarcoma, and ascites hepatomas AH 130 and AH 66.

The principal results obtained were as follows:

1) Regardless of the differences in the kinds of the animal tumors, living hemolytic streptococci caused a definite inhibition of invasion power of the tumor cells in animals.

   Additionally, an appreciable amount of streptolysin S was detected in the supernatant fluids of the six incubation mixtures.

2) In these respects, heat-killed streptococci were, however, entirely without effect.

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Table 1. Anticancer effect shown by a suspending mixture of hemolytic streptococci and Ehrlich carcinoma cells in relation to its incubation temperature

Streptococcal suspension: Hemolytic streptococci, grown in 200 ml of ordinary broth, were washed twice with PBR, and then resuspended in 10 ml of PBR.

Incubation mixtures:

a) A mixture of streptococci and tumor cells was composed of 4 ml of the carcinoma cell suspension (80×10^6 cells/ml of PBR), 8 ml of the streptococcal suspension and 4 ml of PBR.

b) A control mixture was composed of 4 ml of the carcinoma cell suspension and 12 ml of PBR.

Incubation mixture used	Anticancer experiment						
	Temperature of water-bath, in which a mixture of carcinoma cells suspension and cocci suspension was placed for 90 minutes	Animal groups (5 mice for each)	Number of survivors after (days)				
			10	20	30	40	50
Living streptococci suspension + Carcinoma cells suspension	0 °C	A	5	3	2	0	·
	15 °C	B	5	3	0	·	·
	27 °C	C	5	2	1	1	1
	37 °C	D	5	5	5	5	5
Carcinoma cells suspension alone (Control)	0 °C	E	5	2	0	·	·
	15 °C	F	5	3	0	·	·
	27 °C	G	5	2	0	·	·
	37 °C	H	5	2	0	·	·

Table 2. Damaging effect of living hemolytic streptococci on the invasion powor of Ehrlich carcinoma cells in mice

Exptl. group	Mouse		Inoculum	Results		
	No.	Body weight (gm)		Survival day**	Autopsy finding : Positive (+) or negative (−) of tumor invasion in mice	Number of survivors/test animals on the 50th day of inoculation
I	1	17	Living cocci + Carcinoma cells → 37°C, 90′ * (+ Penicillin)	50	−	10/10
	2	17		50	−	
	3	17		50	−	
	4	18		50	−	
	5	18		50	−	
	6	18		50	−	
	7	19		50	−	
	8	19		50	−	
	9	20		50	−	
	10	20		50	−	
II	11	17	Heat-killed cocci (100°C, 30′) + Carcinoma cells → 37°C, 90′ * (+ Penicillin)	16	+	0/10
	12	17		12	+	
	13	17		12	+	
	14	18		11	+	
	15	18		14	+	
	16	18		17	+	
	17	19		13	+	
	18	19		19	+	
	19	20		12	+	
	20	20		14	+	
III (Control)	21	17	Carcinoma cells alone → 37°C, 90′ * (+ Penicillin)	15	+	0/10
	22	17		13	+	
	23	17		17	+	
	24	18		18	+	
	25	18		14	+	
	26	18		18	+	
	27	19		14	+	
	28	19		16	+	
	29	20		16	+	
	30	20		14	+	

* Prior to penicillin addition, an aliquot of the incubated mixture was withdrawn, centrifuged, and the clear supernatant fluid was tested for its hemolytic activity.

** Animals still alived 50 days after inoculation were sacrificed and autopsied.

Table 3. Damaging effect of living hemolytic streptococci on the invasion power of Sarcoma 180 cells in mice

Exptl. group	Mouse		Inoculum	Results		
	No.	Body weight (gm)		Survival day **	Autopsy finding: Positive (+) or negative (−) of tumor invasion in mice	Number of survivors/test animals on the 50th day of inoculation
I	1	17	Living cocci + Sarcoma cells → 37°C, 90′ * (+ Penicillin)	50	−	9/10
	2	17		50	−	
	3	17		50	−	
	4	17		50	−	
	5	17		50	−	
	6	17		48	+	
	7	18		50	−	
	8	18		50	−	
	9	18		50	−	
	10	18		50	−	
II	11	17	Heat-killed cocci (100°C, 30′) + Sarcoma cells → 37°C, 90′ * (+ Penicillin)	16	+	0/10
	12	17		17	+	
	13	17		14	+	
	14	17		18	+	
	15	17		18	+	
	16	17		18	+	
	17	18		19	+	
	18	18		14	+	
	19	18		15	+	
	20	18		17	+	
III (Control)	21	17	Sarcoma cells alone → 37°C, 90′ * (+ Penicillin)	21	+	0/10
	22	17		21	+	
	23	17		20	+	
	24	17		21	+	
	25	17		22	+	
	26	17		18	+	
	27	18		19	+	
	28	18		18	+	
	29	18		17	+	
	30	18		19	+	

Table 4. Damaging effect of living hemolytic streptococci on the invasion power of Leukemia SN 36 cells in mice

Exptl. group	Mouse No.	Mouse Body weight (gm)	Inoculum	Results: Survival day **	Results: Autopsy finding: Positive (+) or negative (−) of tumor invasion in mice	Results: Number of survivors/test animals on the 50th day of inoculation
I	1	21	Living cocci + Leukemia cells → 37°C, 90′ → * (+Penicillin)	50	−	5/10
	2	18		50	−	
	3	21		32	+	
	4	21		22	+	
	5	22		50	−	
	6	20		14	+	
	7	20		36	+	
	8	20		23	+	
	9	19		50	−	
	10	20		50	−	
II	11	18	Heat-killed cocci (100°C, 30′) + Leukemia cells → 37°C, 90′ → * (+ Penicillin)	8	+	0/10
	12	18		8	+	
	13	18		9	+	
	14	19		9	+	
	15	19		12	+	
	16	20		13	+	
	17	20		9	+	
	18	20		10	+	
	19	19		10	+	
	20	20		11	+	
III (Control)	21	20	Leukemia cells alone → 37°C, 90′ → * (+ Penicillin)	14	+	0/10
	22	18		9	+	
	23	20		8	+	
	24	19		8	+	
	25	19		9	+	
	26	20		9	+	
	27	20		8	+	
	28	21		10	+	
	29	22		10	+	
	30	20		9	+	

Table 5. Damaging effect of living hemolytic streptococci on the invasion power of Yoshida sarcoma cells in rats

Exptl. group	Rat No.	Rat Body weight (gm)	Inoculum	Results: Survival day **	Results: Autopsy finding: Positive (+) or negative(−) of tumor invasion in rats	Results: Number of survivors/test animals on the 50th day of inoculation
I	1	120	Living cocci + Yoshida sarcoma cells → 37°C, 90′ * → (+ Penicillin)	50	−	4/10
	2	130		50	−	
	3	127		50	−	
	4	102		27	+	
	5	112		25	+	
	6	140		26	+	
	7	115		23	+	
	8	95		15	+	
	9	129		32	+	
	10	126		50	−	
II	11	137	Heat-killed cocci (100°C, 30′) + Yoshida sarcoma cells → 37°C, 90′ * → (+ Penicillin)	10	+	0/10
	12	109		11	+	
	13	104		11	+	
	14	120		12	+	
	15	127		12	+	
	16	127		12	+	
	17	130		11	+	
	18	115		11	+	
	19	110		12	+	
	20	140		10	+	
III (Control)	21	129	Yoshida sarcoma cells alone → 37°C, 90′ * → (+ Penicillin)	12	+	0/10
	22	142		10	+	
	23	125		11	+	
	24	125		11	+	
	25	130		10	+	
	26	124		15	+	
	27	120		10	+	
	28	119		10	+	
	29	107		11	+	
	30	139		12	+	

* Prior to penicillin addition, an aliquot of the incubated mixture was withdrawn, centrifuged, and the clear supernatant fluid was tested for its hemolytic activity.

** Animals still alived 50 days after inoculation were sacrificed and autopsied.

Table 6. Damaging effect of living hemolytic streptococci on the invasion power of ascites hepatoma AH 130 cells in rats

Exptl. group	Rat No.	Rat Body weight (gm)	Inoculum	Results: Survival day **	Results: Autopsy finding: Positive (+) or negative (−) of tumor invasion in rats	Results: Number of survivors/test animals on the 50th day of inoculation
I	1	62	Living cocci + AH 130 cells → 37°C, 90′ * (+ Penicillin)	50	−	4/10
	2	61		15	+	
	3	63		50	−	
	4	60		15	+	
	5	62		17	+	
	6	63		50	−	
	7	62		14	+	
	8	61		18	+	
	9	60		14	+	
	10	64		50	−	
II	11	60	Heat-killed cocci (100°C, 30′) + AH 130 cells → 37°C, 90′ * (+ Penicillin)	17	+	0/10
	12	62		14	+	
	13	63		12	+	
	14	60		16	+	
	15	61		12	+	
	16	64		11	+	
	17	60		16	+	
	18	63		12	+	
	19	62		12	+	
	20	61		13	+	
III (Control)	21	61	AH 130 cells alone → 37°C, 90′ * (+ Penicillin)	13	+	0/10
	22	62		16	+	
	23	60		14	+	
	24	64		14	+	
	25	63		12	+	
	26	62		18	+	
	27	60		19	+	
	28	61		12	+	
	29	64		12	+	
	30	62		13	+	

Table 7. Damaging effect of living hemolytic streptococci on the invasion power of ascites hepatoma AH 66 cells in rats

Exptl. group	Rat		Inoculum	Results		
	No.	Body weight (gm)		Survival day **	Autopsy finding: Positive (+) or negative (−) of tumor invasion in rats	Number of survivors/test animals on the 50th day of inoculation
I	1	98	Living cocci + AH 66 cells → 37°C, 90′ * (+ Penicillin)	50	−	7/10
	2	100		21	+	
	3	112		50	−	
	4	108		50	−	
	5	105		50	−	
	6	100		50	−	
	7	102		21	+	
	8	104		25	+	
	9	104		50	−	
	10	102		50	−	
II	11	100	Heat-killed cocci (100°C, 30′) + AH 66 cells → 37°C, 90′ * (+ Penicillin)	12	+	0/10
	12	102		15	+	
	13	100		12	+	
	14	110		13	+	
	15	105		18	+	
	16	104		12	+	
	17	106		12	+	
	18	102		16	+	
	19	101		14	+	
	20	100		12	+	
III (Control)	21	98	AH 66 cells alone → 37°C, 90′ * (+ Penicillin)	14	+	0/10
	22	99		13	+	
	23	105		16	+	
	24	100		11	+	
	25	102		13	+	
	26	102		13	+	
	27	106		13	+	
	28	105		13	+	
	29	106		13	+	
	30	107		13	+	

Table 8. Estimation of streptolysin S produced in tumor cells-living hemolytic streptococci mixtures

Exptl. series	An aliquot of sample withdrawn from a incubated (37°C, 90 min.) mixture of			Highest dilution causing hemolysis of supernatant of the mixture	cf. table
I′	Ehrlich carcinoma cells	plus	Living cocci	1: 32	2
II′		plus	Heat-killed cocci	1: <2	
III′		plus	Control (without cocci)	1: <2	
I′	Sarcoma 180 cells	plus	Living cocci	1: 32	3
II′		plus	Heat-killed cocci	1: <2	
III′		plus	Control (without cocci)	1: <2	
I′	Leukemia SN 36 cells	plus	Living cocci	1: 16	4
II′		plus	Heat-killed cocci	1: <2	
III′		plus	Control (without cocci)	1: <2	
I′	Yoshida sarcoma cells	plus	Living cocci	1: 32	5
II′		plus	Heat-killed cocci	1: <2	
III′		plus	Control (without cocci)	1: <2	
I′	Ascites hepatoma (AH 130) cells	plus	Living cocci	1: 32	6
II′		plus	Heat-killed cocci	1: <2	
III′		plus	Control (without cocci)	1: <2	
I′	Ascites hepatoma (AH 66) cells	plus	Living cocci	1: 32	7
II′		plus	Heat-killed cocci	1: <2	
III′		plus	Control (without cocci)	1: <2	