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Antitumor Activity of Clostridial Cell Components

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Cell components of ***Clostridium*** sp. were tested for their antitumor activity, when given to ddN-mice by the intraperitoneal injections 10 times (2 times before and 8 times after the implantation of sarcoma-180). Extracellular insoluble polysaccharide (galactose : glucose = 13.5 : 86.5) produced by ***Clostridium*** sp. No. ***45*** completely regressed the growth of sarcoma-180 in 17 of 20 mice. Lyophilized cells of C. ***saccharoperbutylacetonicum*** regressed in 7 of 10 mice. Cell walls of C. ***saccharoperbutylacetonicum, C. acetobutylicum*** and C. ***butylicum*** showed the complete regression ratios of 18, 17 and 20/20, respectively. The activity of cell wall derived mainly from peptidoglycan rather than polysaccharide layer. Acid- and alkali-extract of fresh cells of C. ***saccharoperbutylacetonicum*** showed the intensive antitumor activity. The acid-extract completely regressed in 10/10 and all mice survived over 100 days.

INTRODUCTION

Clostridium saccharoperbutylacetonicum was used for industrial butanol fermentation (Hongo and Murata, 1965). On the other hand, antitumor activity of bacterial cells or cell components of BCG (Davignon ***et al.,*** 1970; Gutterman ***et al., 1974;*** Mathé ***et al., 1969;*** Rosenthal ***et al.,*** 1972), ***C.*** *parvum* (Halpern ***et al.,*** 1966), ***S. pyogenes*** (Koshimura ***et al.,*** 1964 ; Okamoto ***et al.,*** 1967), ***Mycobacterium*** (Azuma ***et al.,*** 1974; Ogura ***et al.,*** 1975; Tokuzen ***et al.,*** 1975, 1978; Yoshimoto ***et al., 1976)*** and ***L.*** *bulgaricus* (Bogdanov ***et al.,*** 1975) has been reported. The present work is concerned with an antitumor activity of cells or cell components as novel utilizations of anaerobic bacteria.

MATERIALS AND METHODS

Screening of extracellular polysaccharide producing strain

PG medium was used throughout this screening. It consisted of extract of potato, 150 g; glucose, 10 g; ammonium sulfate, 1 g and calcium carbonate, 3g per liter. Bacterial growth was at 30°C for about 3 days under reduced atmospheric pressure (5 to 10 mmHg).

Culture of *Clostridium* sp. No.45

Clostridium sp. **No.45** was cultured in a modified TYA medium. It con-

sisted of glucose, 40g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; $FeSO_4 \cdot 7H_2O$, 6 mg; ammonium acetate, 3 g ; polypepton (Daigo Eiyo Kagaku Ltd.), 2 g and deionized water, 1,000 ml. The pH was adjusted to 6.5 with NaOH and autoclaved for 15 min at 116°C. Fresh medium was inoculated with sufficient organisms (10% seed) and placed at 30°C for 2 days with cotton plug.

Preparation of extracellular polysaccharide

The precipitates of culture broth were washed 3 times with cold deionized water. Extracellular polysaccharide was dissolved into dimethyl sulfoxide and separated from the cells by centrifugation. It was precipitated with 80 % ethanol, washed 5 times with deionized water, then dialyzed against deionized water and finally lyophilized.

Cultures of *C. saccharoperbutylacetonicum*, C. *acetobutylicum*, and C. *butyticum*

Cultural conditions were the same as those of *Clostridium* sp. **No.45** except the cultural time. C. *saccharoperbutylacetonicum*, *C. acetobutylicum* and C. *butylicum* were cultivted for about 6, 12 and 12 hr, respectively, to obtain exponentially growing cells (0. D. 660=0.5-O. 7).

Preparation of chemical- or heat-treated cells

The cells were harvested and washed 3 times with cold deionized water before each treatment. For acetone treatment, the cells were washed twice with 10 volumes of acetone and then diethyl ether. Precipitates were dried under reduced pressure. For formalin treatment, the cells were treated with 4% formalin with mixing overnight at room temperature. Precipitates were washed 5 times with deionized water and lyophilized. For heat treatment, the cells were heated either for 10 min at 100°C or for 30 min at 80°C. Precipitates were washed 5 times with deionized water and lyophilized.

Preparation of cell walls

The cell walls of C. *saccharoperbutylacetonicum*, *C. acetobutylicum* and C. *butylicum* were prepared as described by Kawata and Takumi (1971). The cells were harvested in middle logarithmic growth phase, and suspended in cold deionized water. The cell walls were prepared by differential centrifugation, aftet disruption of the cells by sonication for 15 min using Insonator (model ZOOM, Kubota Co.). The resulting cell walls were immediately exposed to 1 % sodium dodecyl sulfate (SDS) solution with stirring at 37°C for 15 hr. The SDS-treated cell walls were washed by the centrifugation 5 times with deionized water and lyophilized.

Preparation of peptidoglycan and polysaccharide from cell wall

Polysaccharide was extracted twice from cell walls with 5 % trichloroacetic acid with stirring at 35°C overnight. The supernatant was dialyzed against deionized water and lyophilized. On the other hand, peptidoglycan was prepared from the precipitate by washing with deionized water by centrifugation and lyophilized.

Preparation of acid-, alkali- or phenol-extract

The cells were harvested and washed 3 times with cold deionized water before each extraction. For acid extraction, the cells were added to 500ml of 0.125 N HCl solution and 1 ml of 40 % HCHO and stirred for 30 min at 70°C. Acid-extract was precipitated with 85% ethanol and the precipitate was dialyzed against deionized water and lyophilized. For alkali extraction, the cells were added to 500ml of 24% KOH containing 15g of H_3PO_4 and stirred overnight at room temperature. Alkali-extract was acidified with HCl and then precipitated with 80% ethanol. The precipitate was dialyzed against deionized water and lyophilized. For phenol extraction, the cells were added to 500ml of 45 % phenol and stirred for 15 min at 65°C. The water layer after centrifugation was dialyzed against deionized water and lyophilized.

Preparation of water-extract

The cells were harvested and washed 3 times with cold deionized water before extraction. The cells were added to 500ml of deionized water containing 1 ml of 40 % HCHO and stirred for 30min at each temperature, from 20 to 100°C. Each water-extracts were precipitated with 85% ethanol, and the precipitates were dialyzed against deionized water and finally lyophilized.

Antitumor bioassay

The procedures of antitumor bioassay were the same as those in an accompanying communication (Hayashida and Watanabe, 1983), except for the sample concentration (5mg/ml unless otherwise stated) in PBS (−) (phosphate buffered saline without Ca^{2+} and Mg^{2+}).

Sugar analysis

The details of sugar analysis by gas-liquid chromatography were described in an accompanying communication (Hayashida and Watanabe, 1983).

RESULTS

Production and autitumor activity of extracellular polysaccharide from *Clostridium* sp.

Among 120 strains of *Clostridium* which were stocked in our laboratory, five strains, No. 4, No. 22, No.45, No.73 and No. 81, produced extracellular high molecular materials. The strain No. 45, which has subterminal spores and does not hydrolyze gelatin, seeming to belong to the group 1 of *Clostridium* by Bergey's manual (Smith and Hobbs, 1974), produced about 2 mg of the polysaccharide per liter of culture broth. Total carbohydrate content of the polysaccharide was 40.2 % as glucose' by anthrone-sulfuric acid method (Koehler, 1952), and the crude protein was 26.0% by Lowry method (Lowry *et al.*, 1951). The sugar composition was 13.5 % galactose and 86.5 % glucose as weight. The tumor completely regressed in 16 or 17 of 20 mice at the dosages of 1 or 2 mg/day with 10 times injection, respectively; the mice sur-

vived over 100 days, whereas the all control mice died for about 23 days after tumor implantation (Table 1).

Antitumor activities of lyophilized C. *saccharoperbutylacetonicum* whole cells

When exponentially growing cells (0. D. =0. 5-O. 7) were harvested, about lg cells as dry cell weight were prepared from 1 liter culture broth. Effects of these lyophilized whole cells of C. *saccharoperbutylacetonicum* on sarcoma-180 are shown in Table 2. All of 10 mice died within about 20 days after tumor implantation. When 1 mg of lyophilized whole cells of C. *saccharoperbutylacetonicum* were administered 10 times, 7 of 10 mice did not get cancer and survived over 100 days after tumor implantation. Three mice died this time, but mean survival days of these mice were *62.7* days after tumor implantation. This shows a strong activity in prolongation of life of these mice died.

Table 1. Effects of extracellular polysaccharide produced by *Clostridium sp. No. 45* on sarcoma-180.

Dose[a] (mg/mouse)	Complete regression[b]	Survival days[c] (mean±SD)
Control	0/20	22.8±3.6
1 x 10	16/20	27.0±9.8
2x10	17/20	35.0±8.2

[a] ddN mice were given intraperitoneal injections of 50 and 100 mg/kg/day of sample in PBS (−) for 10 days. Only PBS (-) was injected as control. Sarcoma-180 cells were also injected intraperitoneally.

[b] Complete regression was measured as the ratio of survivors over 100 days after sarcoma-180 implantation against test mice.

[c] Survival days were estimated as the mean survival days and standard deviation of died mice.

Table 2. Effects of whole cell of *Clostridium saccharoperbutylacetonicum* on sarcoma-MO.

Dose" (mg/mouse)	Complete regression	Survival days (mean ±SD)
Control	0/10	20.2± 4.3
1 x 10	7/10	62.7±27.9

[a] ddN mice were given intraperitoneal injections of 50 mg/kg/day of sample in PBS (−) for 10 days. The other conditions were the same as those in Table 1.

Antitumor activities of chemical- or heat-treated cells of C. *saccharoperbutylacetonicum*

Effects of these chemical- or heat-treated cells of C. *saccharoperbutylacetonicum* are shown in Table 3. All control mice died in about 20 days. With a weak treatment as acetone or heat treatment at 80°C for 30 min, complete

regression (survivors/tested mice on 100 days after tumor implantation) was both 9/10 and the strong antitumor activities were maintained. While a strong treatment as formalin or heat treatment at 100°C for 10 min reduced the antitumor activities.

Antitumor activity of clostridial cell wall

Effects of clostridial cell walls on sarcoma-180 are shown in Table 4. Cell yields of each strain as dry cell weight was almost the same (1 g/liter culture broth), but yields of cell walls were varied significantly. Cell wall of C. *saccharoperbutylacetonicum* was prepared with the largest amount of all (13 mg/g dry weight of cells) and those of C. *acetobutylicum* and C. *butylicum* were 5 and 3 mg/g dry weight of cells, respectively. In each case, all control mice died in about 20 days after tumor implantation. When 1 mg of C. *saccharoperbutylancetonicum* cell wall was administered 10 times, strong effect on the prolongation of life was recognized; 18 of 20 mice did not get cancer, and 2 died mice survived for 56.0 days in average. In the case of C. *acetobutylicum*

Table *3.* Effects of chemical treated cells of *Clostridium saccharoperbutylacetonicum on* sarcoma-180.

Material	Dose[a] (mg/mouse)	Complete regression	Survival days (mean&SD)
Acetone-treated cell	Control	0/10	20.2± 4.3
	1x10	9/10	70.0± 0
	4x10	3/5	66.5±33.5
Formalin-treated cell	Control	0/10	20.2± 4.3
	1×10	5/ 9	25.5± 2.7
	4×10	2/ 4	51.0± 2.0
Heat-treated cell (80°C, 30 min)	Control	0/10	20.2± 4.3
	1x10	9/10	24.0± 0
	4x10	5/ 6	85.0± 0
Heat-treated cell (100°C. 10 min)	Control	0/10	20.2± 4.3
	1 x 10	6/10	33.8±18.7

[a] ddN mice were given intraperitoneal injections of 50 and 200 mg/kg/day of sample in PBS (−) for 10 days. The other conditions were the same as those in Table 1.

Table 4. Effects of *Clostridium* cell wall on sarcoma-180.

Cell wall	Dose[a] (mg/mouse)	Complete regression	Survival days (mean&SD)
C. saccharoperbutylacetonicum	Control	0/20	18.6± 2.0
	1x10	18/20	56.0±20.0
C. acetobutylicum	Control	0/20	19.9± 3.6
	2x10	17/20	61.3±17.5
C. butylicum	Control	0/20	22.0± 3.3
	1x10	20/20	—

[a] ddN mice were given intraperitoneal injections of or 100 mg/kg/day of sample in PBS (−) for 10 days. The other conditions were the same as those in Table 1.

cell wall, complete regression was 17/20 and survived days of died mice was 61.3 days in average. Cell wall of C. *butylicum* had very strong activity; complete regression was 20/20 and tumor growth was almost perfectly inhibited. Effects of polysaccharide and peptidoglycan from cell wall on sarcoma-180 are shown in Table 5. Complete regression by polysaccharide and peptidoglycan were 7 and 17/20, respectively. These results indicate that the antitumor activity of cell wall could exist mainly in peptidoglycan, although polysaccharide had some activity as well.

Table 5. Effects of polysaccharide and peptidoglycan fractions from *Clostridium saccharoperbutylacetonicum cell* wall on sarcoma-180. Dose conditions were mentioned in Table. 2.

Material	Complete regression	Survival days (mean±SD)
Control	0/20	21.9± 2 3
Polysaccharide	7/20	36.6117.6
Peptidoglycan	17/20	78.3f21.5

Table 6. Effects of cell extracts of ***Clostridium saccharoperbutylacetonicum*** on sarcoma-180. Dose conditions were mentioned in Table 2.

Material	Complete regression	Survival days (mean±SD)
Control	0/10	17.4± 2.0
Acid extract	10/10	—
Acid residue	5/10	26.2± 4.8
Alkali extract	9/10	93.0± 0
Alkali residue	8/10	37.0±18.0
Phenol extract	0/10	21.2± 4.8
Phenol residue	8/10	28.5± 2.5

Antitumor activities of acid- and alkali-extract from C. *saccharoperbutylacetonicum*

Yields of acid- and alkali-extract were 67 and 170 mg/g dry weight of cells. Effects of acid-, alkali- and phenol-extract on sarcoma-180 are shownin Table 6. Control mice died in about 18 days. Acid-extract possessed strongest activity and complete regression was 10/10, while the activity of acid-residue was low. In the case of alkali extraction, alkali-extract showed strong activity and alkali-residue had considerable activity as well. In the case of phenol extraction, activity existed in the residue and phenol-extract did not have any activity.

Antitumor activities of water-extracts from C. *saccharoperbutylacetonicum*

Aggregates by ethanol appeared rapidly in water extracts obtained above 60°C, while a little precipitate were found in one day after the addition of ethanol in the extracts obtained below 60°C. Yields of precipitates caused by

Table 7. Effects of water-extracts of *Clostridium saccharoperbutylacetonicum* on sarcoma-180. Dose conditions were mentioned in Table. 2.

Temperature (°C)	Complete regression	Survival days (mean&SD)
Control	0/10	20.2±3.9
40	9/10	90.0±0
60	9/10	62.0±0
	10/10	--
80	9/10	87.0±0
100	9/10	75.0±0

ethanol addition were 43, 85 and 112 mg/g dry weight of cells in the extracts at 20, 60 and 100°C, respectively. The effects of water-extracts on sarcoma-180 are shown in Table 7. Striking inhibitory activities against the growth of tumor cells were recognized in all extracts obtained at 20 to 100°C.

DISCUSSION

As a result of study of antitumor activity of many fermentation bacteria, a strong antitumor activity was recognized in clostridial cells and cell components.

Up to this time, many bacterial components are reported as antitumor substances. Halpern ***et al.*** (1966) reported the antitumor activity of C. *parvum*. BCG is well known as strong antitumor substances and these cells are currently used in a clinical level (Davignon ***et al.,*** 1970; Gutterman ***et al.,*** 1974; Mathé, ***et al.,*** 1969; Rosenthal ***et al.,*** 1972). The antitumor activity of *S. pyogenes* was reported by Koshimura ***et al.*** (1964) and Okamoto ***et al.*** (1967), and penicllin treated cells of S. *pyogenes* (OK-432) are widely used in Japan. Bogdanov ***et al.*** (1975) reported the antitumor activity of cell component of ***L. bulgaricus*** and the active component was found to be peptidoglycan. Yamamura and his colleagues (Azuma ***et al.,*** 1974; Ogura et al., 1975; Tokuzen ***et al.,*** 1975, 1978; Yoshimoto ***et al.,*** 1976) purified partially the cell of BCG (CWS) and recognized the strong antitumor activity almost without toxicity.

Clostridial cell and cell component (extracellular polysaccharide, cell wall, acid- and alkali-extract) showed strong antitumor activity compared with already known substances. Compared with BCG, culture of ***Clostridium*** sp. is easy, cells grow fast, and acetone-butanol fermentation can be carried out in a large scale.

Among cell walls of ***Clostridium*** sp., the cell wall of C. ***butylicum*** showed the strongest activity. Antitumor activity of cell walls existed mainly in peptidoglycan rather than polysaccharide layer. Azuma ***et al.*** (1971) reported the immunological adjubant activity of BCG cell wall was strong in peptidoglycan layer. These are considered to be the same results.

Acid-extract showed in particular strong activity and inhibited perfectly the growth of sarcoma-180 ascite tumor. Water soluble fraction of acid-extract had strong antitumor activity and any toxicity was not recognized.

The chemical structure of acid-extract will be reported in the following paper.

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