Preparation and Antitumor Activities of Mitomycin C $\beta$-(1→6)- Branched (1→3)-$\beta$-D-Glucan Conjugate

Shigeyuki Usui, Kohei Murashima, Miho Sakai, Tadashi Kuro, and Shigeo Ukai*
Gifu Pharmaceutical University, Mitahora-higashi 5-chome, Gifu 502, Japan.
Received March 16, 1994; accepted May 24, 1994.

The conjugate of mitomycin C (MMC) with carboxymethylated schizophyllan (CMSPG) which was prepared from monochloroacetic acid and schizophyllan (SPG), a $\beta$-(1→6)-branched (1→3)-$\beta$-D-glucan from Schizophyllum commune Fries, was synthesized by using 3-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. The degree of substitution of carboxymethyl groups in CMSPG was estimated as approximately 0.87, and locations of carboxymethyl groups in CMSPG were predominantly determined at O-4, O-6, and O-4, 6 positions in glucose residues. The contents of MMC in the conjugate were estimated to be between 8 and 12% (w/w). The conjugate showed successive monoexponential liberation, with a half-life of 7.2 h. Although the in vitro cytotoxicity of the conjugate against L1210 leukemia cells was similar to that of MMC when the cells were exposed for 24 and 48 h, the 50% growth-inhibitory concentration of the conjugate for L1210 was twice times higher than that of MMC with exposure for 12 h. The antitumor activity of the conjugate against subcutaneously implanted sarcoma 180 solid tumor in mice by intraperitoneal (i.p.) administration was similar to that of MMC at a dose of 1.5 mg eq MMC per kg per d for both 7 times of continuous administration and 4 times of intermittent administration. However, the reduction in the number of leukocytes in the peripheral blood, which was the side effect of MMC, was suppressed by the intermittent administration of the conjugate. The conjugate maintained the ability to induce the tumor regressing factor and the neutrophil chemotactic factor in the serum.

Keywords schizophyllan; mitomycin C; conjugate; antitumor activity; tumor regressing factor; chemotactic factor

The limited usage of cancer chemotherapeutic agents prevents the effective therapy due to the side effects and indiscriminate cytotoxicity of these agents. The effectiveness of the pro-drug conjugated cancer chemotherapeutic agents, such as mitomycin C (MMC) and adriamycin with the polysaccharides as a soluble carrier, has been reported as a promising means of more selectively targeting the focal tissues and reducing toxic side effects.1–4 Matsumoto et al. suggested that with the conjugate of MMC with dextran, the pharmacokinetic behavior of MMC might be regulated by chemical modification of the carrier involving molecular weight, ionic charge, and the length of the spacer between MMC and dextran.5 However, the polysaccharides, dextrans and mannans, were used simply as carriers, having no biological activity of their own.

As a possible approach to macromolecular pro-drugs as cancer chemotherapeutic agents, we have previously designated the conjugates of MMC with antitumor polysaccharides.6,7 These conjugates are expected to maintain immunological activity with the polysaccharides and to enhance the effect of the cancer chemotherapeutic agents. A linear (1→3)-$\beta$-D-glucan (PS) from Alcaligenes faecalis var. myxogenes IFO 13140,8 and branched (1→3)-$\beta$-D-glucans such as schizophyllan (SPG),9 lentilman,10,11 and scleroglucan12 have been well studied regarding their structure and antitumor activity. A chemically modified glucan which has been synthesized by the carboxymethylation of PS has been reported to exhibit antitumor activity against sarcoma 180 (S-180) transplanted in mice through a host-mediated immunological action.13–15 We have recently reported the synthesis of a conjugate of MMC with carboxymethylated (1→3)-$\beta$-D-glucan (CMPS), as well as its antitumor activity.16 It has been shown that the MMC-CMPS conjugate maintains the antitumor activity and the immunological activity of CMPS and, furthermore, it suppresses the side effects of MMC, reducing the number of leukocytes in the peripheral blood.

Since the MMC-CMPS conjugate seemed to be less soluble, we further searched for a more soluble polysaccharide with antitumor activity to serve as a carrier. In this paper, we chose SPG as a carrier for anticancer agent, because it is more soluble, and we describe the synthesis of the conjugate-introduced MMC to the carboxymethylated SPG (CMSPG) and the subsequent antitumor activity of the MMC-CMSPG conjugate. In addition, we demonstrate that the MMC-CMSPG conjugate maintains a similar immunological activity to SPG.

MATERIALS AND METHODS

Materials SPG and CMSPG were kindly supplied by Kaken Pharmaceutical Industry Co., Ltd., Tokyo, Japan and Takeda Pharmaceutical Industry Co., Ltd., Osaka, Japan, respectively. MMC was a generous gift from Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan. Sephadex G-10 and Toyopearl HW-65F were obtained from Pharmacia Fine Chemicals and Tohso Manufacturing Co., Ltd., respectively. Other chemicals were purchased commercially at the highest available grade.

Preparation of CMSPG The carboxymethylation of SPG was performed essentially according to the method of Sasaki et al.13) Five hundred mg of SPG was suspended into 13.3 ml of isopropanol. To the suspension, 0.33 ml of 30% NaOH was added 4 times at 15 min intervals and the mixture was stirred at room temperature for 90 min. Then, 0.63 g of monochloroacetic acid was added 3 times at 10 min intervals to the mixture, and the reaction mixture was stirred at 50°C for 150 min and subsequently at room temperature for 16 h. The reaction product was collected...
by low-speed centrifugation and the precipitate was dissolved into 13.3 ml of water. After the solution was neutralized with acetic acid, 22.5 ml of ethanol was added. The precipitate was collected by centrifugation and subsequently washed with, in order, 80% methanol: ethanol (2:1) and 80% ethanol: ether (1:1). After the residual organic solvents were evaporated, the product was dissolved into an appropriate volume of water and then lyophilized.

**Determination of Position and Degree of Substitution of Carboxymethyl Groups in CMSPG** The position and degree of substitution of the carboxymethyl groups in CMSPG were determined by our method described previously. The hydroxymethylated product was prepared by the reaction with CMSPG and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and by subsequent treatment with NaBH₄. The hydrolysis of the hydroxymethylated product was performed by the treatment with, in order, 90% formic acid and 2N trifluoroacetic acid (TFA). After TFA was evaporated, the hydrolysate was dissolved into an appropriate volume of water and then reduced by the addition of NaBH₄. The reduced hydrolysate was dried after the removal of excess NaBH₄ by treatment with cationic Amberlite CG-120 resin followed by repeated evaporation with methanol, and the residual sample was acetylated with pyridine and anhydrous acetic acid. The acetylated sugars were subjected to analysis by gas chromatography-mass spectrometry (GC-MS) (model JMS-D300 mass spectrometer, JEOL). GC-MS was operated at 200°C using a glass column (0.2 cm x 1 m) packed with 2% EGSS-X. The tendency of the helical conformation of CMSPG was measured by the reported method using aniline blue.

**Preparation of MMC–CMSPG Conjugate** The MMC–CMSPG conjugate was prepared by the method reported previously, using MMC and CMSPG instead of CMPS. To a solution of 50 mg of CMSPG in 30 ml of water was added 200 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. The mixture was stirred at 15°C for 30 min, and then adjusted to about pH 6.0 with diluted HCl. Fifteen mg of MMC was added to the mixture with pH values maintained between 5.7 and 6.0 with diluted HCl, and the mixture was stirred at 15°C for 24 h. After the mixture was neutralized by aqueous sodium bicarbonate and dialyzed against water at 4°C for 4 d, the MMC–CMSPG conjugate was obtained by lyophilization. The glucan and MMC contents in the conjugate were estimated by the phenol–sulfuric acid method and the absorbance at 364 nm, respectively, after aliquots of the lyophilized conjugate were dissolved in distilled water. To confirm the binding of MMC and CMSPG, the mixture of the conjugate and MMC was applied onto a Sephadex G-10 column (1.5 x 30 cm) equilibrated with 0.9% NaCl. Two ml aliquots of fractions were collected, and each fraction was measured by the absorbance at 364 nm.

**Gel Filtration Chromatography** For determination of the molecular weight, 2 mg of the polysaccharide samples was dissolved in 0.5 ml of 0.3 M NaCl and applied onto a Toyopera HW-65F (1.5 x 90 cm) equilibrated with 0.3 M NaCl, and then subsequently eluted with the same solution at a flow rate of 8 ml/h. Two ml aliquots of the fractions were collected and the glucan was estimated by the phenol–sulfuric acid method. The molecular weight standards used were Dextran T-110 (105000), Dextran T-250 (253000), and Dextran T-500 (495000) (Pharmacia Fine Chemicals).

**In Vitro Release Experiment** Ten ml of the MMC–CMSPG conjugate dissolved at 60 g of MMC equivalent/ml in phosphate buffered saline was incubated at 37°C with moderate shaking. A 1 ml aliquot of the solution was withdrawn and the liberated MMC was separated by ultrafiltration (Sartorius Centrisart I SM 13249). The amounts of MMC released and remaining in the conjugate were estimated by HPLC on a TSK gel ODS-120T column (4.6 x 150 mm) using 35% methanol and by measuring the absorbance at 364 nm, respectively.

**In Vitro Cytotoxicity Assay** Cell growth inhibition by MMC alone and MMC–CMSPG conjugate was measured using L1210 leukemia cells in RPMI 1640 medium supplemented with 10% fetal bovine serum and containing streptomycin (100 μg/ml), penicillin (100 units/ml), and 0.05% 2-mercaptoethanol. Cells were seeded on a 96-well microplate at a density of 5 x 10⁴ cells/well and the culture was added the medium containing the test materials at various concentrations. This was followed by incubation in a humidified atmosphere with 5% CO₂ at 37°C for the period indicated. The cytotoxicity of the samples was assayed by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method reported previously.

**In Vivo Antitumor Assay** S-180 ascites cells were used in the assay for the antitumor activities of MMC, CMSPG, and the MMC–CMSPG conjugate. Cells were commonly obtained by sequential intraperitoneal (i.p.) passage into ddY male mice (Japan SLC Inc., Shizuoka, Hamamatsu). S-180 cells were transplanted subcutaneously at a dose of 2 x 10⁶ cells per 0.1 ml into the right groin of ddY male mice (4 weeks old). Two separate schedules for administration of the test samples were planned. One was that the test samples dissolved into sterile saline were continuously administered i.p. once a day for 7 d at a dose of 1.5 mg of MMC equivalent/kg, starting from 1 d after transplantation of S-180. The other was that administration of the test samples was done at a dose of 1.5 mg of MMC equivalent/kg on days 7, 10, 13, and 16 after transplantation. Tumor growth was observed for 30 d, then the mice were killed and the tumors were extirpated and weighed. The inhibition ratios were calculated using the formula: inhibition ratio (%) = [(A - B)/A] x 100, where A is the average tumor weight of the control group, and B is that of the tested group.

**Measurement of the Number of Leukocytes in Mice** Twenty μl of the blood of the tumor-bearing mice were taken from the orbital vein with a heparinized haemocrit tube at 1 d after the final administration. The blood was diluted with Isoton II (Coulter Scientific, Japan), hemolyzed with Zap-oglobin II (Coulter Scientific, Japan), and the leukocytes were counted by an automatic blood cell counter MEK-3100 (Nihon Kohgen Inc.).

**Assay for Induction of Tumor Regressing Factor** The assay was performed according to the method described by Kunimoto et al. ICR female mice (6 weeks old)
bearing 14-d-old S-180 tumors received an i.p. injection of each sample (CMSPG 100 mg/kg, MMC-CMSPG conjugate 100 mg eq CMSPG/kg, MMC 13 mg/kg) dissolved in sterile saline, with sterile saline only for the control group. Blood was collected 14–16 h after the administration, kept on ice for 30 min, and the serum was prepared by centrifugation. All sera were stored at −40 °C until use. The sera were injected intravenously (i.v.) into S-180 bearing ICR female mice (6 weeks old) on day 14 of tumor growth. Three mice were used for each sample. The tumors were extirpated 24 h later, minced, and incubated at 37 °C for 2 h with stirring in an enzyme mixture containing 0.125% trypsin, 0.1% collagenase, and 0.02% deoxyribonuclease I in Eagle’s minimum essential medium using a volume of 20 times the tumor weight. In the resulting cell suspensions, the total number of tumor cells and other cells was determined by differential counting of fixed and stained preparations under a binocular microscope, or by viable tumor cell counting under a phase-contrast microscope.

**Assay for Neutrophil Chemotactic Activity** The assay was carried out in accordance with the description by Boyden.\(^2\) ICR female mice (6 weeks old) received an i.p. injection of polysaccharide and MMC samples at a dose of 100 mg eq CMSPG/kg. The blood was collected 12 and 24 h after administration. The serum was prepared after the blood was kept on ice for 30 min and stored at −40 °C until use. Polymorphonuclear leukocytes (PMN) were prepared by the reported method with ultracentrifugation at 60000 × g for 20 min in isotonic Percoll solution (d = 1.110 g/ml), and were suspended into RPMI 1640 medium supplemented with 10% fetal calf serum.\(^2\) The fraction containing more than 98% of PMNs was used for chemotaxis assay. The serum (25 μl) was poured into the lower chamber of a 48-well microplate chemotaxis chamber (Neuro Probe, U.S.A.) and a filter with a 5 μm pore size (type PFB 5, Neuro Probe) was layered on it. Fifty μl of the PMN suspension (1 × 10⁶ cells/ml) was applied onto the upper chamber and the chamber was incubated in a humidified atmosphere with 5% CO₂ at 37 °C for 30 min. After the migrated PMNs on the lower side of the filter were fixed with methanol and stained with Giemsa’s solution, the number of PMNs were counted under a binocular microscope.

### RESULTS AND DISCUSSION

Starting from 500 mg of SPG, 680 mg of CMSPG was obtained by the carboxymethylation of SPG with 7.5% monochloroacetic acid in isopropanol containing 5.6% NaOH. The introduction of carboxymethyl groups to SPG was confirmed by the IR spectrum of CMSPG on which the signal was shown at approximately 1600 cm⁻¹ (data not shown). The position of carboxymethyl groups in glucose residues of CMSPG was elucidated by the method developed in our laboratory.\(^1\) The procedure involves the reduction of carboxymethyl groups to O-hydroxyethyl groups, hydrolysis of the resulting O-hydroxyethyl SPG, and GC-MS analysis of the hydrolysate as alditol acetates. As shown in Table I, 4-O-hydroxyethyl-glutitol of the product from monohydroxymethyl substituent acetate was obtained with the highest yield (28.3%). The derivatives from dissubstituents such as 4, 6-Dihydroxyethyl-glutitol acetate (10.8%) and 1, 2-O-ethylene-6-O-hydroxyethyl-α-D-glucopyranose (9.5%) were gained with higher yield. These results suggest that the carboxymethyl groups are predominantly substituted at the O-4 and/or O-6 position in glucose residues of SPG. The degree of substitution of carboxymethyl groups in CMSPG was estimated as approximately 0.87. The average molecular weight of CMSPG was estimated to be approximately 440000 by gel filtration, suggesting that CMSPG was almost not degraded to a smaller molecular weight by carboxymethylation because the average molecular weight of SPG is reported to be about 450000.\(^9\) However, the fluorescence intensity based on the formation of the complex with CMSPG and aniline blue was diminished greater than that with SPG and aniline blue (Table II). These results suggest that the high-ordered structure of the triple helix in CMSPG is largely destroyed; nevertheless, there is no change in the molecular weight between CMSPG and SPG.

MMC was conjugated to CMSPG using the watersoluble carbodiimide of 1-ethyl-3-(dimethylaminopropyl)carbodiimide by controlling the pH between 5.7 and 6.0 in the reaction mixture. The contents of MMC in the MMC–CMSPG conjugate was estimated to be between 8 and 12% (w/w) by measuring the absorbance at 364 nm for MMC and by the phenol-sulfuric acid method for glucan. Sephadex G-10 column chromatography was carried out for confirming the linkage of MMC and

### Table I. Patterns of Carboxymethyl Substitution of Glucose Residue in CMSPG

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glc</th>
<th>2-CM-Glc</th>
<th>4-CM-Glc</th>
<th>6-CM-Glc</th>
<th>2,4-diCM-Glc</th>
<th>2,6-diCM-Glc</th>
<th>4,6-diCM-Glc</th>
<th>Degree of substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMSPG</td>
<td>37.6</td>
<td>4.6</td>
<td>28.3</td>
<td>4.8</td>
<td>4.4</td>
<td>9.5</td>
<td>10.8</td>
<td>0.87</td>
</tr>
</tbody>
</table>

Glc, Glucose; 2-CM-Glc, 2-O-carboxymethyl-D-glucose; 4-CM-Glc, 4-O-carboxymethyl-D-glucose; 6-CM-Glc, 6-O-carboxymethyl-D-glucose; 2,4-diCM-Glc, 2,4-di-O-carboxymethyl-D-glucose; 2,6-diCM-Glc, 2,6-di-O-carboxymethyl-D-glucose; 4,6-diCM-Glc, 4,6-di-O-carboxymethyl-D-glucose.

### Table II. Changes in Fluorescence Intensities of Aniline Blue with Polysaccharides

<table>
<thead>
<tr>
<th>Sample</th>
<th>Relative fluorescence intensity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPG</td>
<td>100</td>
</tr>
<tr>
<td>CMSPG</td>
<td>2.4</td>
</tr>
<tr>
<td>CMPS</td>
<td>92.7</td>
</tr>
<tr>
<td>Dextran T-250</td>
<td>0.1</td>
</tr>
</tbody>
</table>
CMSPG in the prepared conjugate (Fig. 1), indicating that MMC is attached to CMSPG. The in vitro release rate of MMC from the MMC–CMSPG conjugate was measured in the phosphate buffered saline (pH 7.4) at 37°C (Fig. 2). The conjugate showed successive monoeponential liberation, with a half-life of 7.2h. Although the release rate of MMC from the MMC–CMSPG conjugate was slower than that from the MMC–CMPS conjugate (4.75h), the rate was much faster than that from the MMC–dextran conjugate synthesized by Kojima et al. The different release rates of MMC from various conjugates could depend on the length and composition of the spacer between MMC and the carrier of polysaccharides, as reported by Sezaki. Since CMSPG, which is more soluble than CMPS, has been chosen as a carrier, the CMSPG–MMC conjugate has the advantage of great solubility and long-term storage without a loss of MMC from the conjugate after lyophilization. On the other hand, it was difficult for the CMPS–MMC conjugate reported previously to redissolve with an aqueous solution after lyophilization and, therefore, the CMPS–MMC conjugate should be stored in an aqueous solution below freezing to avoid the degradation of the conjugate.

In vitro cytotoxicities of MMC and the MMC–CMSPG conjugate were assayed using L1210 mouse leukemia cells with various exposure periods in the culture (Table III). Although the 50% growth-inhibitory concentration (IC_{50}) of the conjugate was two times higher than that of MMC when the cells were exposed for 12h, the cytotoxicity of the conjugate was similar to that of MMC for 24 and 48h. This result indicates that MMC is not completely released from the conjugate for 12h of exposure because it has a half-life of 7.2h and the cytotoxicity of MMC is masked under the conjugate and revealed by its release from the conjugate.

The antitumor activities of MMC and the MMC–CMSPG conjugate against S-180 solid tumor in mice were tested at a dose of 1.5 mg MMC equivalent/kg with two separate plans of administrations. One was continuous administration every day for 7d after S-180 was transplanted (Table IV), and the other was intermittent administration every 3d, 10d after transplantation of S-180 (Table V). Strong inhibition, of more than 90% of

---

Table III. In Vitro Cytotoxicity of MMC and MMC-CMSPG Conjugate against L1210 Cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC_{50} (MMC eq µg/ml)</th>
<th>12h</th>
<th>24h</th>
<th>48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMC</td>
<td>7.69</td>
<td>4.58</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>MMC–CMSPG conjugate</td>
<td>16.81</td>
<td>5.69</td>
<td>0.73</td>
<td></td>
</tr>
</tbody>
</table>

a) IC_{50} test material concentration showing 50% growth inhibition.

Table IV. Antitumor Activity of MMC-CMSPG Conjugate and MMC by Continuous Administration against S-180

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose (MMC eq µg/kg/d x 7)</th>
<th>Mean tumor wt. ± S.E. (g)</th>
<th>Inhibition ratio (%)</th>
<th>Complete regression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>9.56 ± 1.36</td>
<td>—</td>
<td>0/9</td>
</tr>
<tr>
<td>CMSPG</td>
<td>12.3</td>
<td>5.31 ± 1.20</td>
<td>44</td>
<td>0/7</td>
</tr>
<tr>
<td>MMC</td>
<td>1.5</td>
<td>0.91 ± 0.58</td>
<td>90</td>
<td>1/7</td>
</tr>
<tr>
<td>MMC + CMSPG mixture</td>
<td>1.5 ± 12.3</td>
<td>0.77 ± 0.37</td>
<td>92</td>
<td>2/9</td>
</tr>
<tr>
<td>MMC–CMSPG conjugate</td>
<td>13.8</td>
<td>0.25 ± 0.15</td>
<td>97</td>
<td>5/9</td>
</tr>
</tbody>
</table>

a) MMC-CMSPG conjugate contents 10.9% MMC. Significant difference from control; b) p<0.05, c) p<0.01.

Table V. Antitumor Activity of MMC-CMSPG Conjugate and MMC by Intermittent Administration against S-180

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose (MMC eq µg/kg/d x 4)</th>
<th>Mean tumor wt. ± S.E. (g)</th>
<th>Inhibition ratio (%)</th>
<th>Complete regression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>10.96 ± 1.25</td>
<td>—</td>
<td>0/7</td>
</tr>
<tr>
<td>CMSPG</td>
<td>15.7</td>
<td>6.07 ± 1.43</td>
<td>43</td>
<td>1/7</td>
</tr>
<tr>
<td>MMC</td>
<td>1.5</td>
<td>3.50 ± 1.78</td>
<td>67</td>
<td>1/7</td>
</tr>
<tr>
<td>MMC + CMSPG mixture</td>
<td>1.5 ± 15.7</td>
<td>3.90 ± 1.11</td>
<td>63</td>
<td>1/7</td>
</tr>
<tr>
<td>MMC–CMSPG conjugate</td>
<td>17.2</td>
<td>2.45 ± 0.93</td>
<td>77</td>
<td>1/7</td>
</tr>
</tbody>
</table>

a) MMC-CMSPG conjugate contents 8.7% MMC. Significant difference from control; b) p<0.05, c) p<0.01.
the tumor growth by MMC alone, by the MMC and CMSPG mixture, and by the MMC–CMSPG conjugate and, especially, the complete regression of the tumor transplanted in 5 out of 9 mice by the conjugate were observed with the continuous administration. The conjugate also showed a better effect on tumor growth than either MMC alone or the mixture, although only moderate inhibitions of 63–77% by MMC alone, the mixture, and the conjugate were observed with the intermittent administration.

Since the antitumor activity of the MMC–CMSPG conjugate appeared to be slightly better than others tested, the side effect of MMC on the number of leukocytes in peripheral blood\(^{25}\) was investigated. In the continuous administration of MMC alone, the MMC and CMSPG mixture, or the MMC–CMSPG conjugate, the numbers of leukocytes in the peripheral blood on the day after the final administration were significantly reduced to 50–63% compared with the control group (Table VI). However, the number of leukocytes did not significantly decrease by giving the conjugate in the intermittent administration; nevertheless, significant reductions to 57 and 58% against the control group were observed by injecting MMC alone and the mixture, respectively (Table VII). In addition, the number of leukocytes in the intermittent administration of the conjugate was significantly different \((p<0.01)\) from that in the mixture. These results suggest that the conjugate has the advantage of reducing the side effect of MMC without changing the antitumor activity by the intermittent administration.

For further investigation of biological activities maintained by MMC–CMSPG conjugate as an immunological response modifier, the induction of the tumor regressing factor reported by Kunimoto \(et\ al.\)\(^{15}\) and the chemotactic activity for polymorphonuclear leukocytes were assayed. The cellular composition in tumor lumps carried by mice which were given an i.v. injection of the sera prepared from the tumor-bearing mice, which were administrated i.p. injections of MMC alone, the MMC and CMSPG mixture, and the MMC–CMSPG conjugate, are shown in Fig. 3. Although the group administered MMC alone showed no change in cellular composition compared with the control group, which was given the serum prepared by the injection of saline, the numbers of S-180 cells decreased to less than half of the control group by the injections of the sera prepared from the administrations of CMSPG, the mixture, and the conjugate, similar to the injection of the serum prepared from the administration of CMPS, which has been reported to be a carboxymethyl derivative of the \((1\rightarrow3)\)β-D-glucan inducing the tumor regressing activity by Kunimoto \(et\ al.\)\(^{15}\). This result suggests that the conjugate still maintains the activity of inducing the tumor regressing factor in the serum.

Although Kunimoto \(et\ al.\)\(^{15}\) have reported that polymorphonuclear leukocytes accumulate in the tumor lump by the i.v. injection of the serum containing the tumor regressing factor, whether the accumulation of polymorphonuclear leukocytes is based on the chemotactic activity of the serum is unknown. Since the tumor
regressing activity has been found in the serum prepared by the administration of CMSPG and the conjugate, we further studied the chemotactic activity of the serum for the polymorphonuclear leukocytes (Table VIII). The neutrophil chemotactic activity was found in the serum prepared 12 h after the injection of CMPS, SPG, CMSPG, and the MMC and CMSPG mixture, but not in the serum from the MMC–CMSPG conjugate. However, 24 h later, the activity was found in the serum from the conjugate just as in CMPS, SPG, CMSPG, and the mixture. This result suggests that antitumor polysaccharides tested are able to induce chemotactic activity for polymorphonuclear leukocytes in the serum after administrations for 12—24 h, and the induction of the chemotactic activity may result from releasing MMC in the case of the conjugate, although it is still unclear whether a single chemotactic factor is induced in the serum treated with polysaccharides or the chemotactic activity is caused by the tumor regressing factor.

REFERENCES