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Immunostimulating Activity of a Crude Polysaccharide Derived from Green Tea (*Camellia sinensis*) Extract

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Green tea extract is well-known to reduce the risk of a variety of diseases. Here, we investigated the immunostimulating activity of tea polysaccharide (TPS), one of the main components in green tea extract. The water extracts from mature or immature tea leaves were precipitated by using ethanol at room temperature. The sediment was washed with ethanol and acetone alternately and then dried. We used the phagocytic activity of macrophage-like cells as an indicator of immune function activation. Chemical components were analyzed by HPLC. The immunostimulating activity of TPS from immature leaves extract was higher than that of TPS from mature leaves, and its activities were dependent on the content of strictinin in the leaf extract. Futhermore, a mixture of catechin and TPS that removed polyphenols did not increase the immunostimulating activity. These results suggest that the catechin−polysaccharide complex is a very important molecule in the immunomodulating activity of tea extracts.

KEYWORDS: Green tea extract; crude polysaccharide; immunostimulating activity; strictinin; catechins

INTRODUCTION

Tea, from the plant *Camellia sinensis* L., is one of the most popular beverages consumed worldwide in its green, black, or oolong form. It contains many compounds such as polyphenols, polysaccharides, amino acids, vitamins, and so on, and it reduces the risk of a variety of diseases (*1*). Tea polyphenols and tea polysaccharides are the main components in tea extracts. Epigallocatechin gallate (EGCG), a major polyphenolic compound in tea extracts, is well-known to have antioxidant (*2*), antibacterial (*3*), and immunomodulatory effects (*1, 4*), as well as antitumor, anticarcinogenic, and antimutagenic activities. Tea polysaccharides are also reported to have blood-glucose-reducing (*5*) and antioxidant effects (*6*) and the ability to inhibit pathogenic bacterial adhesion (*7*). Wang et al. (*8*) reported that tea polysaccharides enhanced immunization of rats. In general, it is known that food polysaccharides in mushrooms, algae, and plants activate macrophage immune responses and lead to immunomodulation, antitumor activity, wound healing, and so on (*9*); however, there are still only few reports on the immunostimulating effect of tea polysaccharides, and furthermore, the dependence of immunostimulating activity on the level of leaf maturation has never been reported.

In this study, we found that the immunostimulating activity of crude polysaccharide from immature tea leaves was higher than that of TPS from mature tea leaves, and we conclude that the catechin−polysaccharide complex is a potential immunostimulator.

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MATERIALS AND METHODS

Preparation of TPS. Tea cultivars were harvested at the plantation of the National Institute of Vegetable and Tea Science in Kanaya, Shizuoka, Japan. Two cultivars (A and B) were chosen from these cultivars. Tea leaves were dried in a microwave oven, pulverized, and stored at 4 °C before analysis. The pulverized tea leaves were boiled 10 times in distilled water (DW) for 30 min and centrifuged for 30 min (1200*g*). The tea extract was treated with polyvinylpolypyrrolidone (PVPP, Sigma, St. Louis, MO) to remove excess phenolic compounds. The PVPP-treated extract was precipitated by using ethanol (final 70% concentration) at room temperature. The sediment was washed with ethanol and acetone alternately and dried (crude polysaccharide, TPS). Separation of high- and low-molecular weight TPS fractions was performed by a dialysis membrane (Slide-A-Lyzer 20 k MWCO, Pierce, Rockford, IL) and a centrifugal filter device (Microcon YM-100, Millipore Co., Bedford, MA, USA) (TPS-F1 and TPS-F2), respectively.

To remove the substances that conjugated with TPS from buds, such as polyphenols and proteins, 100 mg of precipitate was dissolved in 10 mL of 0.1 M NaOH; 1 mL of a NaClO solution (available chlorine >5%) was added. The mixture was then incubated at 4 °C overnight (*10*). The reaction mixture was dialyzed (Slide-A-Lyzer 20 k MWCO, Pierce) with DW, precipitated by using acetone (final 70% concentration) at room temperature, and dried. A total of 100 mg of precipitate was dissolved in 10 mL of DW and centrifuged for 30 min (1200*g*), and this solution was divided into a clear supernatant (TPS-F3) and brown precipitate. The clear supernatant was precipitated by using ethanol (final 70% concentration) at room temperature. The sediment was washed with ethanol and acetone alternately and dried.

Catechin and Strictinin Assays. Chemical components were analyzed by the procedure of Maeda-Yamamoto et al. (*11*). The tea was diluted 3- to 5-fold with DW, and 20 μL of the filtrate after filtration through a membrane filter (DISMIC-13-HPPTFE, pore size 0.45 μm,

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ADVANTEC, Tokyo, Japan) was injected by an autosampler (SIL-10Avp, Shimadzu, Kyoto, Japan) into the HPLC apparatus (Shimadzu Class VP HPLC system). HPLC was performed with a Shimadzu LC-10A pump coupled with a UV detector (SPD-M 10Avp, Shimadzu) by using a reverse-phase Wakopak Navi C18-5 column (4.6 mm i.d. × 150 mm, 5 μm granule diameter, Wako Pure Chemical Industries, Ltd., Osaka, Japan) with a Wakopak Navi C18-5 column (4.6 mm i.d. × 10 mm, 5 μm granule diameter, Wako Pure Chemical Industries, Ltd.) as a guard column. The solution was then eluted with the eluent described below at a flow rate of 1 mL/min at 40 °C. Strictinin, epicatechin (EC), catechin (C), EGCG, epicatechin gallate (ECG), catechin gallate (CG), and epigallocatechin-3-*O*-(3-*O*-methyl) gallate (EGCG3″-Me) were measured at 272 nm and epigallocatechin (EGC) at 242 nm.

HPLC analysis was performed by using a linear gradient system with a mobile phase A (H_2O−acetonitrile−H_3PO_4, 400:10:1) and a mobile phase B (methanol−mobile phase A, 1:2). Linear gradient elution was performed as follows: 100% mobile phase A for 2 min; 20% mobile phase A for 27 min; 20% mobile phase A for 10 min; and 100% mobile phase A for 7 min.

Quantification was carried out by using the external standard method. Quantification of catechins was performed after data acquisition by using an LC workstation (Class VP system, Shimadzu).

Molecular Weight of TPS. The molecular weight of tea polysaccharides was determined by HPLC equipped with a refractive index detector (RID-10A, Shimadzu) and a Shodex OHpak SB-806 M HQ column (8.0 mm × 300 mm). The elutions of the standard molecular weight of dextran (Pharmacia AB, Uppsala, Sweden) and pullulans (Showa Denko Co. Ltd., Tokyo, Japan) were carried out in the same manner.

Phagocytosis Assay. The phagocytosis assay was performed as previously described (*12*). Briefly, to differentiate cells along the monocytic pathway, HL60 cells (American-type Culture Collection, Rockville, MD) were cultured in RPMI 1640 plates(Gibco-Invitrogen, Burlington, ON, Canada) supplemented with 120 nM VD3 (Wako Pure Chemical Industries, Ltd.). Differentiated HL60 cells were seeded in 48-well plates (BD Biosciences, Franklin Lakes, NJ). Cells were treated with 25 μL of TPS, a catechin mixture (polyphenone E, Mitsui-Norin Co., Ltd., Shizuoka, Japan), or lipopolysaccharide (LPS, Calbiochem, San Diego, CA). Next, 25 μL of a 1% suspension of YG-labeled microspheres (Polysciences, Inc., Warrington, PA) was added, and the solution was incubated at 37 °C in 5% CO_2 for 16 h. The cells were fixed with 2% formaldehyde and resuspended in phosphate-buffered saline. The rate of phagocytosis was measured with an EPICSXL-flow cytofluorometer (Beckman Coulter, Fullerton, CA).

Statistical Analysis. All data were derived from at least three experiments. Data are expressed as the means ± standard deviation (SD). The efficiency of phagocytosis was compared by using the paired *t*-test. A value of $p < 0.05$ was considered significant. The parameter *n* represents the number of experiments.

RESULTS

Immunostimulation Activity of TPS. Phagocytosis by macrophages is used as an important indicator of immune function activation; thus, we estimated the immunostimulation activity of TPS by using VD3-differentiated HL60 cells in which phagocytosis is activated by various immune-enhancing polysaccharides. TPS was classified according to the part of the leaf used (**Figure 1**). The ALL consisted of 10–15% buds and first leaves, 15–20% second leaves, 25–35% third leaves, 25–30% fourth leaves, 10–20% fifth leaves, and 20–25% stems. **Figure 2**, top panel, shows the difference in immunostimulating activity of TPS from the ALL of cultivars A and B in different years. The immunostimulating activity of the TPS changed over the years. On the other hand, **Figure 2**, bottom panel, shows the difference in immunostimulating activity of TPS from each leaf of cultivar A (2007). The immunostimulating activity was

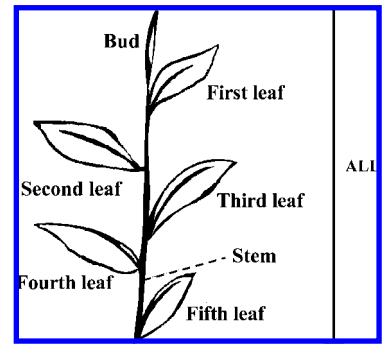

Figure 1. Tea leaf order.

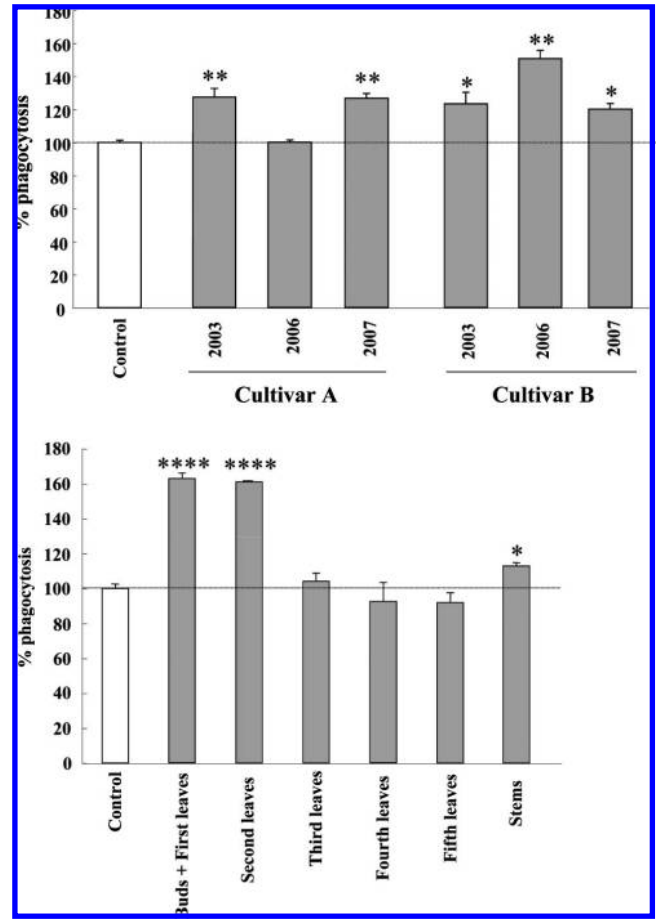

Figure 2. Phagocytic activities of TPS. TPS from the ALL of cultivar A or B (top). TPS from each leaf of cultivar A (bottom). VD3-differentiated HL60 cells were incubated with beads in the presence of TPS (100 μg/mL). Phagocytosis activity in the absence of TPS (control) is normalized to 100%. Values are the means ± SD, $n = 3$. * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$ versus the control.

increased by TPS from buds and first leaves ($p < 0.0001$), second leaves ($p < 0.0001$), and stems ($p < 0.05$); however, TPS from third, fourth, and fifth leaves did not activate phagocytosis.

Polyphenol Content and Immunostimulating Activity. **Table 1** shows the content of total catechin and strictinin in the extracts from the ALL of cultivars A and B, and **Table 2** shows the content of total catechin and strictinin in the extracts from each leaf of cultivar A (2007). In the ALL, a good correlation was obtained between the percentage of phagocytosis and total catechin, but not strictinin (**Figure 3**); however, no correlation was obtained between the percentage of phagocytosis

Table 1. Content of Total Catechin and Strictin in Extracts from the ALL

sample (year)	total catechin[a,b]	strictinin[b]
cultivar A (2003)	13.91 ± 0.008	0.80 ± 0.06
cultivar A (2006)	13.22 ± 0.163	1.48 ± 0.02
cultivar A (2007)	13.70 ± 0.163	1.40 ± 0.02
cultivar B (2003)	13.83 ± 0.157	0.69 ± 0.01
cultivar B (2006)	14.95 ± 0.322	1.03 ± 0.02
cultivar B (2007)	13.10 ± 0.344	0.94 ± 0.02

[a] Total catechin: EC, C, EGCG, ECG, CG, EGC, and EGCG3″-Me. [b] g/100 g of dried leaf.

Table 2. Content of Total Catechin and Strictinin in Extracts from Each Leaves

sample[a]	total catechin[b,c]	strictinin[c]
buds and first leaves	12.72 ± 0.47	3.41 ± 0.13
second leaves	15.37 ± 0.16	2.64 ± 0.07
third leaves	15.88 ± 0.31	1.72 ± 0.05
fourth leaves	14.96 ± 0.05	1.08 ± 0.01
fifth leaves	14.97 ± 0.21	0.76 ± 0.01
stem	10.45 ± 0.02	0.43 ± 0.01

[a] Extract of cultivar A. [b] Total catechin: EC, C, EGCG, ECG, CG, EGC, and EGCG3″-Me. [c] g/100 g of dried leaf.

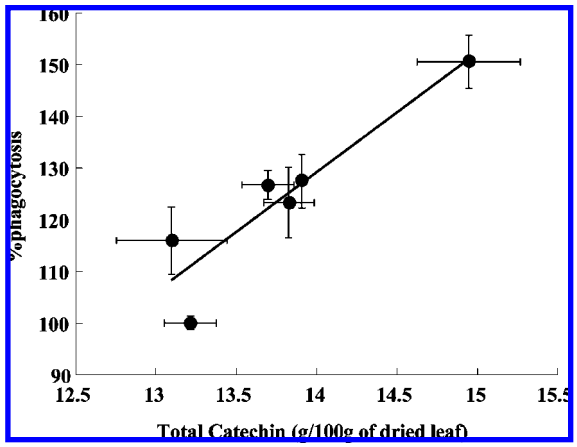

Figure 3. Relationship between the percentage of phagocytosis and total catechin. The data in **Table 1** and **Figure 2** (top panel) were used to construct the relationship. $Y = 23.075X - 194.14$ and $r^2 = 0.8505$, where Y and X are the percentage of phagocytosis and the total catechin, respectively.

and each catechin, such as EGCG. On the other hand, in each leaf, a good correlation was obtained between the percentage of phagocytosis and strictinin (**Figure 4**).

Molecular Weight Distribution of TPS. The molecular weight of TPS is calculated by using the molecular standard fitting equation. The exponential regression equation was $Y = (1.4431e + 13)\ e^{-2.2698X}$, where Y is the molecular weight and X is the retention time. The correlation coefficient was $r^2 = 0.9985$. **Figure 5** shows the molecular weight distribution of TPS from the ALL with immune-stimulating activity. TPS has three major peaks of around 200, 1, and 0.1 ($\times 10^4$) M.W. (**Figure 5A**). Although data are not shown, TPS from buds also showed the same distribution. TPS was separated into two fractions (TPS-F1 and TPS-F2) by a dialysis membrane and a centrifugal filter device (**Figure 5B** and **C**), and the phagocytic activities were measured. TPS-F1 ($p < 0.001$ or 0.05), but not TPS-F2, significantly increased the phagocytic activity (**Figure 6**). The activity of TPS and TPS-F1 was saturated at a final concentration of 100 μg/mL, and their activities were the same at concentrations of 100 μg/mL and higher (data not shown).

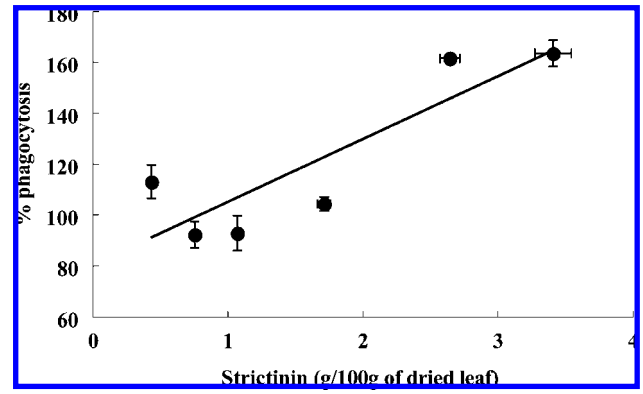

Figure 4. Relationship between the percentage of phagocytosis and strictinin. The data in **Table 2** and **Figure 2** (bottom panel) were used to construct the relationship. $Y = 24.678X - 79.619$ and $r^2 = 0.7577$, where Y and X are the percentage of phagocytosis and the strictinin, respectively.

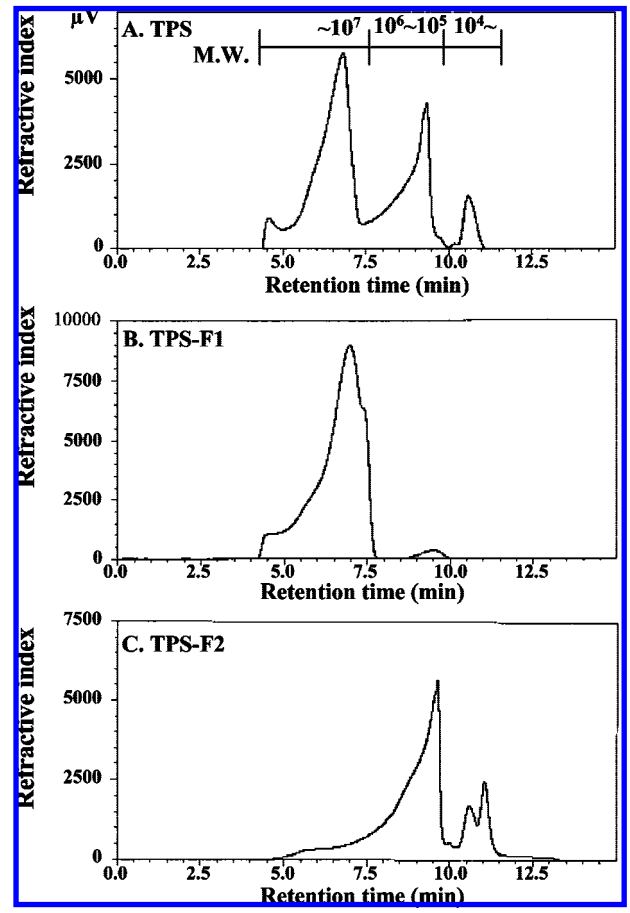

Figure 5. Chromatogram for TPS at a concentration of 10 mg/mL. (**A**) TPS. (**B**) TPS with a molecular weight greater than 10 000 (TPS-F1) was separated by dialysis in DW. (**C**) TPS with a molecular weight less than 10 000 (TPS-F2) was separated by a centrifugal filter device. The third peak (11 min) corresponds to debris from the filter device.

Immunostimulating Activity of TPS That Removed Polyphenols (TPS-F3) and the Brown Precipitate. Figure 7 shows the immunostimulating activity of TPS-F3 and the brown precipitate. Neither TPS-F3 nor the brown precipitate stimulated VD3-differentiated HL60 cells.

Immunostimulating Activity of the Catechin Mixture. Figure 8 shows the immunostimulating activity of the catechin mixture (polyphenone E, green tea catechin extract; the catechin content was more than 90%, and the EGCG content was more

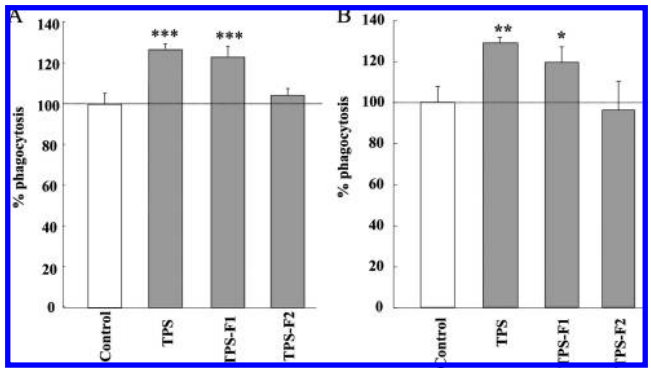

Figure 6. Phagocytic activities of TPS with different molecular weights. (**A**) TPS from the ALL of cultivar A. (**B**) TPS from buds of cultivar A. VD3-differentiated HL60 cells were incubated with beads in the presence of TPS (100 μg/mL). Phagocytosis activity in the absence of TPS (control) is normalized to 100%. Values are the means ± SD, $n = 3$. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus the control.

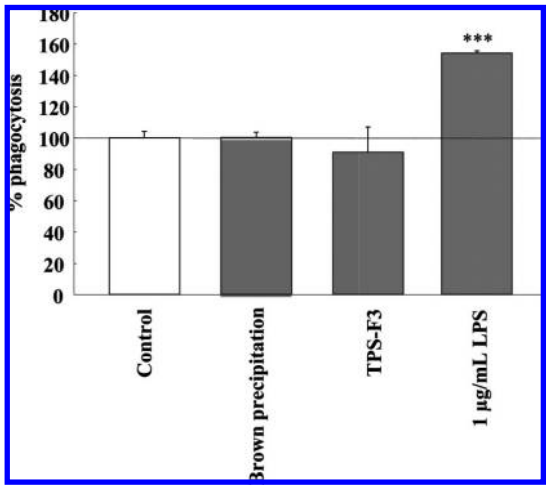

Figure 7. Phagocytic activities of TPS that removed catechins (TPS-F3) and the brown precipitate. VD3-differentiated HL60 cells were incubated with beads in the presence of TPS-F3 (100 μg/mL), the brown precipitate (100 μg/mL), or LPS (1 μg/mL, positive control). The phagocytosis activity in the absence of TPS (control) is normalized to 100%. Values are the means ± SD, $n = 3$. *** $p < 0.001$ versus the control.

than 60%). The catechin mixture did not stimulate VD3-differentiated HL60 cells.

DISCUSSION

In this study, we found that the immunostimulating activity of TPS from buds to second leaves was higher than that of TPS from other leaves (**Figure 2**, bottom panel), and its activity was dependent on the content of strictinin in the leaf extract (**Figure 4**). On the other hand, the immunostimulating activity in all leaves was dependent on the content of total catechin in the leaf extract (**Figure 3**). As shown in **Figure 6**, high-molecular-weight TPS was the main substance with an immunostimulating activity. It is known that strictinin promotes the formation of a catechin−polysaccharide complex (*13*), and there is a lot of strictinin in immature leaves (*14*). As shown in **Figure 4**, strictinin had a threshold concentration (around 2 g/100 g of dried leaf) for the effect. These results suggest that the catechin−polysaccharide complex is a potential immunostimulator, and strictinin is a promoter in the formation of catechin−polysaccharide complex.

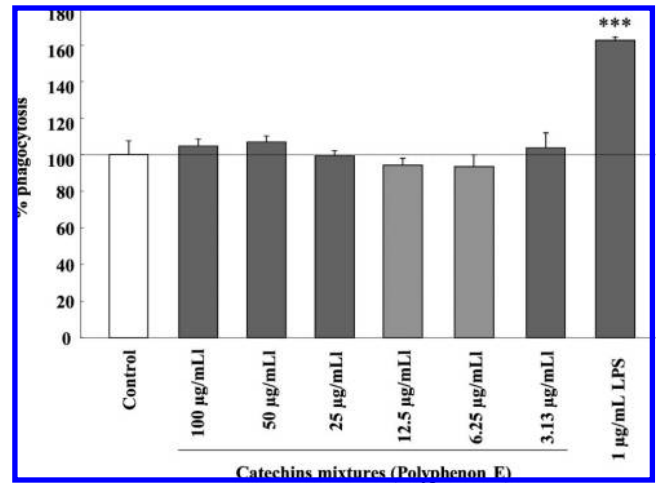

Figure 8. Phagocytic activities of a catechin mixture. VD3-differentiated HL60 cells were incubated with beads in the presence of a catechin mixture or LPS (1 μg/mL, positive control). The phagocytosis activity in the absence of TPS (control) is normalized to 100%. Values are the means ± SD, $n = 3$. *** $p < 0.001$ versus the control.

Tea polysaccharides have been reported to have an immunomodulatory effect (*15*) and the ability to inhibit pathogenic bacterial adhesion (*7*). These previous studies showed that the content of uronic acid in TPS was related to the effects. TPS was found to be mainly composed of uronic acids, especially galacturonic acid. There was more water-soluble pectine in immature leaves than in mature leaves (*16*). TPS in this study was also mainly composed of uronic acids (data not shown); however, TPS that removed polyphenols (TPS-F3) did not stimulate VD3-differentiated HL60 cells (**Figure 7**). Also, tea catechins have been reported to have immunomodulatory effects (*1, 4*), but in this study, a catechin mixture (polyphenon E) did not stimulate VD3-differentiated HL60 cells (**Figure 8**). The immunomodulatory mechanism of the catechin−polysaccharide complex in this study may be different from the mechanism of tea polysaccharides or catechins in previous studies. Nakamura et al. (*17*) reported that a complex mixture of tannins with polyphenols and polysaccharides inhibited tumor promotion and carcinogenesis in mice and mice cell lines. These results were similar to ours. However, the mechanism underlying these activities is still not clear. Phagocytic activity is mediated by phagocytic receptors that can be categorized into different structural classes such as C-type lectin receptors, leucine-rich repeat receptors, Ig superfamily members, scavenger receptors, and so on (*18*). Phagocytic receptors recognize the molecular features of immunostimulators. These phagocytic receptors may be associated with the immunostimulating activity of the catechin−polysaccharide complex.

The present study demonstrates that the crude polysaccharide from tea leaf containing a lot of catechins is a potential immunostimulator, and strictinin might promote the formation of a catechin−polysaccharide complex. Catechins are the main polyphenols in green tea extract. This suggests that the catechin−polysaccharide complex is a very important molecule in the immunomodulating activity of tea extracts. Further studies and data are needed to clarify the mechanism of immunomodulation by catechin−polysaccharide complexes.

ABBREVIATIONS USED

TPS, tea polysaccharide; VD3, 1,25-dihydroxyvitamin D3; EGCG, epigallocatechin gallate; EGC, epigallocatechin; ECG,

epicatechin gallate; EC, epicatechin; C, catechin; CG, catechin gallate; EGCG3″-Me, epigallocatechin-3-*O*-(3-*O*-methyl) gallate.

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