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Generation of Cytotoxic Material to Murine Spleen Cells by Periodate Oxidation and Borohydride Reduction of Pectic Substances

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Periodate oxidation–borohydride reduction (IO_4/BH_4) is a quite general modification method in carbohydrate chemistry to elucidate structure and function. Upon IO_4/BH_4 treatment, pectins from citrus and apple and pectic acid gave macromolecular modification products that were cytotoxic to spleen cells *in vitro*. On the other hand, the same treatment of a neutral polysaccharide, dextran, produced no cytotoxicity. These findings suggest that when active sites of carbohydrates are determined based on the results of these treatments, careful interpretation is necessary.

Keywords——pectin; spleen cell; polyanion; toxic compound; periodate oxidation

The Smith degradation, involving periodate oxidation and borohydride reduction (IO_4/BH_4), has been applied extensively in the structural elucidation of polysaccharides and glycoconjugates. Recently, this treatment has often been used for determining the structural requirements of a carbohydrate moiety for activity in biologically active substances.

During the characterization of the physicochemical properties of Tohki mitogen,[1] which was obtained from *Angelica actiloba* KITAGAWA (Yamato Tohki) by hot water extraction, and shows potent murine immunomodulating activity, such as mitogenicity, adjuvanticity, interferon-inducing activity, macrophage activation, and antitumor activity,[2] it was found that the main components were pectic substances.[1] We also used IO_4/BH_4 treatment of the mitogen and observed the disappearance of the mitogenicity after the treatment.[1] However, various data suggested that some cytotoxic material had been generated in the modification reaction.[1] In the present paper, we describe the formation of cytotoxic materials from pectins, as model compounds, by IO_4/BH_4 treatment.

We used three types of pectins (from apple and citrus and polygalacturonic acid) with a dextran as a reference compound. These pectins and dextran were oxidized with sodium metaperiodate in acetate buffer (pH 5.5) and 4 °C. The reaction was terminated by the addition of ethylene glycol and then the reaction mixture was dialyzed. The resulting non-dialyzable solutions were reduced to polyols with sodium borohydride in the usual way, and then dialyzed and concentrated. The effects of these materials on murine spleen cells were determined by proliferation assay, based on the incorporation of [^{3}H]thymidine (TdR) into deoxyribonucleic acid (DNA). Lipopolysaccharide (LPS) from *E. coli* and concanavalin A (Con A) were usually used as positive mitogenic controls.[3a)]

As shown in Fig. 1, in the case of dextran (Fig. 1-D, F), [^{3}H]TdR incorporation was not inhibited at any dose tested, either with or without LPS or Con A. IO_4/BH_4 treatment of dextran had little effect. These observations suggest that neither native nor IO_4/BH_4-treated dextran possesses cytotoxicity, and that the IO_4/BH_4 products of other neutral polysaccharides might also be non-mitogenic. However, in the case of IO_4/BH_4-treated pectin, [^{3}H]TdR incorporation was decreased at concentrations higher than 25 μg/well both with and without LPS or Con A, although native pectin showed cytotoxicity. These observations

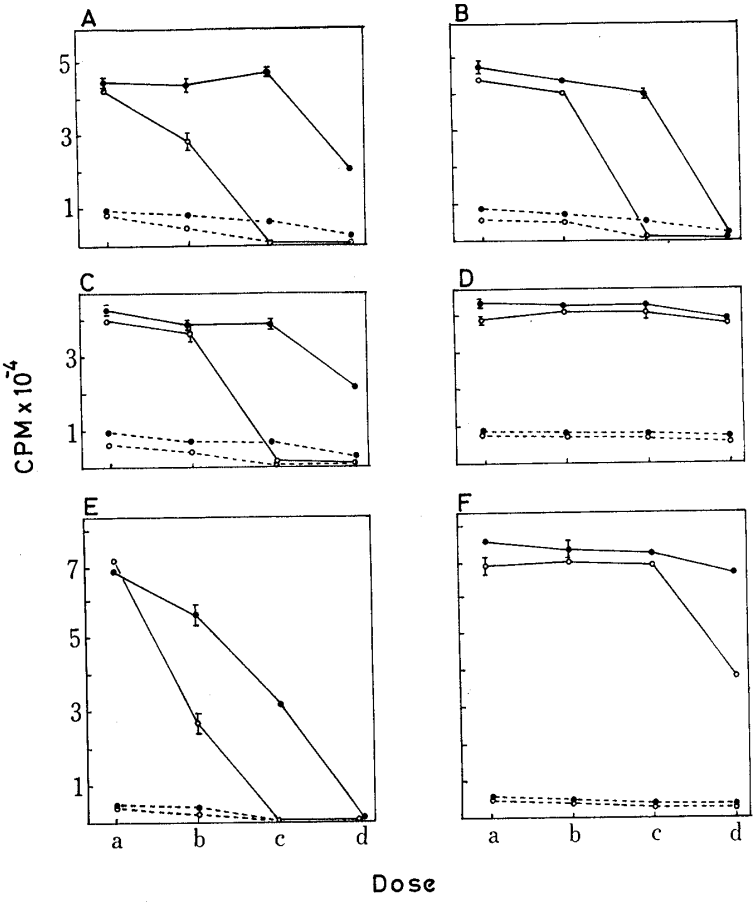

Fig. 1. Effect of Pectin on the [^{3}H]TdR Incorporation of Spleen Cells

Solid line, with stimulants (A—D, LPS 0.25 μg/culture; E, F, Con A 0.625 μg/culture); dotted line, without stimulant; closed circles, native polysaccharides; open circles, IO_4/BH_4-treated polysaccharides. A, Pectic acid; B, Pectin from apple; C, Pectin from citrus; D, Dextran; E, Pectic acid; F, Dextran. a, 1 μg/culture; b, 5 μg/culture; c, 25 μg/culture; d, 125 μg/culture; Con A only, 86649 ± 1060 cpm.

indicate that cytotoxicity was generated by IO_4/BH_4 treatment in the case of pectin. This could lead to erroneous interpretation in research on the active sites of biologically important materials.

Why did the IO_4/BH_4-treated products from polyuronides, but not dextran, show cytotoxicity? It has been noted generally that aldehyde groups generated by IO_4 oxidation may cause either positive or negative immune stimulation.[4] However, in this work, aldehyde groups were derivatived to polyols by subsequent BH_4 reduction. It is known that polyuronides such as alginate form inter- or intra-ring bonds with adjacent residues during IO_4 oxidation,[5] and the formation of such bonds between aldehyde and other anionic groups might allow intact carbonyl groups to survive after BH_4 reduction of pectin.

Toxic materials generated by IO_4/BH_4 treatment of pectins are anionic, and this activity did not disappear after further treatment with BH_4. Biopolymers such as immunomodulators (LPS,[3a] Con A,[3a] glucan[3b]), peptide hormones (insulin[6]) and toxins[7] act on cells through receptor molecules on the cell surface. On the other hand, the mechanisms of action of macromolecular toxins can be classified into several groups such as 1) enzymic action,[8] 2) membrane-damaging action,[9] and 3) neurotoxic action,[10] and IO_4/BH_4-treated pectins may fall into the second group. Polyanionic substances such as LPS, dextran sulfate and vesiculogen show various immunomodulating activities.[11] However, pectin shows no such action and IO_4/BH_4-treated pectin shows cytotoxicity. Thus, polyanionic substances show

various actions on cells, and further research on the structure–activity relationships should be fruitful.

Materials and Methods

Materials——Pectin from citrus and from apple, sodium metaperiodate, sodium borohydride were purchased from Wako Pure Chemical Ind. Ltd. Pectic acid (polygalacturonic acid) was purchased from Nakarai Chemicals Ltd. Dextran T 2000 and Con A were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). LPS from *E. coli* 055 B5 was purchased from Difco Laboratories (Detroit, Mich.).

Periodate Oxidation and Borohydride Reduction——A representative treatment schedule was as follows: pectin, pectic acid or dextran was dissolved in 60 ml of 50 mM acetate buffer (pH 5.5), and 210 mg of sodium metaperiodate was added. The reaction mixture was allowed to stand in the dark at 4 °C for 96 h. Then ethylene glycol (1 ml) was added, and the mixture was allowed to stand for 1 h to destroy the excess periodate, and dialyzed against water. Non-dialyzable material was reduced with 210 mg of sodium borohydride for 2 h. The excess borohydride was destroyed by acidification with acetic acid, and the reaction mixture was dialyzed against water. The non-dialyzable fraction was concentrated *in vacuo* and lyophilized.

Proliferation Assay——Mice (C3H/HeN) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu, Japan. Lymphocyte suspensions were prepared from spleens by teasing the organs in the cold medium. The cell suspensions were passed through a 200 gauge stainless steel sieve and then allowed to stand to precipitate tissue fragments. The supernatant containing single cells was separated, and centrifuged (600 ***g*** for 5 min). The cells were resuspended gently in 0.83% ammonium chloride solution to lyze red cells and washed with fresh medium. RPMI 1640 medium (Nissui Seiyaku Co., Ltd., Tokyo, Japan) containing HEPES (5 mM), penicillin G (100 U/ml), and streptomycin (100 U/ml) was used in these treatments. After being washed three times with the medium, the cells were suspended at 5×10^6 viable cells per ml (counted by the Trypan blue dye exclusion method) in the above medium containing 7.5% heat inactivated fetal calf serum (FCS, GIBCO, Grand Island, New York, U.S.A.). Aliquots (25 μl) were placed in flat-bottomed microtiter plates (Nunclon 163320, Nunc, Denmark) and two-fold enriched medium (25 μl) was added to stabilize the osmotic pressure, then the cell suspension (100 μl) prepared above was added to each well. The final assay conditions were 5×10^5 cells in 150 μl of RPMI 1640 medium containing HEPES, penicillin G, streptomycin, and 5% fetal calf serum (FCS) per well. Each culture plate was incubated at 37 °C for 48 h in a humidified atmosphere of 5% CO_2–95% air. Before harvesting, cultures were pulsed for 20 h with 0.5 μCi of tritiated TdR (20 μl) (NET-355, [6-^{3}H]TdR; New England Nuclear, Boston, Massachusetts, U.S.A.) dissolved in the above medium. The cultures were harvested with a Labo Mash multiple cell harvester (Labo Science Co., Ltd., Tokyo, Japan) on glass fiber filters. TdR incorporated into the cells was determined with a liquid scintillation counter (Aloka 903, Tokyo, Japan) with an Omnifluor–toluene scintillator. Results were expressed as arithmetic mean cpm $\pm$ S.D. of triplicate cultures.

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