Application of the popliteal lymph node assay (PLNA) for evaluation of the antigenicity of water-soluble food colors

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Abstract

The mouse popliteal lymph node assay (PLNA) has been proposed as an immunotoxicological test to predict the allergenicity of chemicals without additional adjuvant. Although a PLN response in the primary PLNA is also observed in association with non-specific activation induced by some irritants, the PLN response in the secondary PLNA in previously sensitized animals is used to detect memory immune responses without using adjuvant.

In this study PLNA was applied to evaluate the antigenicity of water-soluble food colors. The PLN cellularity index of an individual dye was calculated from the cell count of exposed and control PLNs of mice. The xanthene dyes Food Red No.3, Food Red No.104, and Food Red No.105, but not Food Red No.106 gave a high PLN cellularity index. The azo dyes and triphenylmethane dyes were not associated with any increase in PLN cellularity index. Indigo Carmine (Food Blue No.2) also had a high PLN cellularity index. The reactivity of the PLNA correlated well with the chemical structure category of the synthetic dyes. The high PLN cellularity index was considered to be associated with protein binding.

Key words: popliteal lymph node assay; PLNA; LLNA; synthetic dye; food additive

I. Introduction

The testing method for the antigenicity of food additives has not been fully established. Acute systemic anaphylaxis (ASA) and passive cutaneous anaphylaxis (PCA) have been used to test the antigenicity of medical supplies, but they are not suitable for detection of the antigenicity of low-molecular weight supplies. The local lymph node assay (LLNA) is widely used as an antigen detection method. In the LLNA an antigenic substance is applied to a mouse pinna, and the reactivity is observed. Since it is hard to retain water-soluble test solution at a mouse pinna, the improved method for applying LLNA to some water-soluble substances has been reported\(^1\). Recently the abdominal wall method (AW method) using the mouse abdominal wall and adjuvant has been used to detect anaphylactic reactions\(^2\).

The popliteal lymph node assay (PLNA) was initially proposed as a method for detection of systemic autoimmune reactions\(^3\), and later also as a method of assessing the immunostimulating and sensitizing potentials of drugs without additional adjuvant\(^4\). Although a popliteal lymph node (PLN) response in the primary PLNA is also observed in association with non-specific activation induced by some irritants, the PLN response in the secondary PLNA in previously sensitized animals is used to detect memory immune responses without using adjuvant.

The present study was designed to evaluate the antigenicity of the synthetic dyes as water-soluble materials in vitro by the PLNA, since the synthetic dyes used as food additives are highly water-soluble. The relationship between the PLN cellularity index and the classification of the synthetic dyes was investigated.

In Japan, 83 synthetic dyes (tar dyes) have been approved for use as coloring agents in cosmetics, and Ordinance No.55 (December 13, 1972) of the Ministry of Health, Labour and Welfare classifies the synthetic dyes into three categories from the stand-
point of safety. The dyes in Group I can be used in any pharmaceuticals or cosmetics, and they can also be used as food additives (The Japanese Standards of Food Additives, 7th Edition, The Ministry of Health, Labour and Welfare). In this study, the dyes in Group I were used.

II. Materials and Methods

1. Animals
Female 6-week-old BALB/c mice were purchased from Charles River (Atsugi, Japan) and housed under controlled conditions. Mice were acclimated for a week and were given standard laboratory chow (MF, Oriental Yeast Co., Osaka) and water ad libitum. The experiments in this study were carried out in accordance with "the Ethical Guidelines for Animal Care, Handling, and Termination" of our institute. Five animals were used to determine the PLN cellularity index for each synthetic dye.

2. Reagents
The synthetic dyes permitted as food additives in Japan were used in this study. The standard products distributed by the National Institute of Health Sciences were used. Food Red No.3 (Erythrosine), Food Red No.104 (Phloxine B), Food Red No.105 (Rose Bengal), and Food Red No.106 (Acid Red) were used as xanthene dyes; Food Red No.2 (Amaranth), Food Red No.102 (New Coccine), Food Yellow No.4 (Tartrazine), Food Yellow No.5 (Sunset Yellow) as azo dyes; Food Green No.3 (Fast Green FCF) and Food Blue No.1 (Brilliant Blue FCF) as triphenylmethane dyes; and Food Blue No.2 (Indigo Carmine) as an indigoid dye. Other chemicals were of reagent grade or of the highest grade commercially available.

3. PLNA
Each dye was dissolved in water and subcutaneously injected in a volume of 50 μl into the left hind footpads of mice with a 26G needle. The pH of each dye solution (10 mg/ml) was 6.8-7.3 except Food Green No.3 (pH 6.0) and Food Red No.2 (pH 8.4). The mice were secondarily immunized 12 days later, by injection of the same dose, except special description, into the same footpads. Two days after secondary immunization, the mice were sacrificed by cervical dislocation. Both left and right PLNs were carefully removed and dissociated in saline. The cell suspensions obtained were filtered through a cell strainer (Becton Dickinson Company, Franklin Lakes, NJ), and the lymphocytes were counted with an automatic cell counter (K-4500, Sysmex, Kobe, Japan). The PLN cellularity index of each mouse was defined as the ratio of the cell count in the exposed left PLN to that in the control right PLN.

The animal experiment in the PLNA is depicted in Fig. 1. In every test, TNBS (2,4,6-trinitrobenzenesulfonic acid; first injection, 1 mg/mouse; second injection, 0.1 mg/mouse) or BSA (bovine serum albumin; 0.5 mg/mouse) was used as positive control compound, and their cellularity indices were confirmed to be almost the same as in previous reports.

III. Results and Discussion

Since the PLN cellularity index of the secondary response to allergic compounds shows a maximum on day 2 after the second immunization, the mice in this study were sacrificed on 2 day after the secondary immunization, and their PLNs were carefully extracted. In the mice whose left hind footpad was subcutaneously injected with TNBS as an allergenic positive control, the secondary response yielded a PLN cellularity index of 13-16. The secondary response was consistent with the report that TNBS showed the maximal PLN cellularity index of 13.1 (first injection, 1 mg/mouse; second injection, 0.1 mg/mouse). In the mice in which BSA was used as a positive control, the PLN cellularity indexes in the tests ranged from 2.0 to 2.5.

Figure 2 shows the PLN cellularity index obtained with xanthene dyes including Food Red No.3, Food Red No.104, Food Red No.105, and Food Red No.106. Xanthene dyes containing halogen substituents, especially Food Red No.104 and Food Red No.105, had significantly higher PLN cellularity indexes. To investigate a dose-response effect, three doses (0.05, 0.5 and 5 mg/mouse) of dyes were injected. At the 5 mg/mouse dose of Food Red No.3, Food Red No.104, and Food Red No.105, the footpads were swollen red and became hard, and necrosis of the heels was observed in some mice. When necrosis was observed, the PLN cellularity index was not calculated.

Xanthene dyes have been reported to bind to BSA. In our study, injected xanthene dyes including Food Red No.3, Food Red No.104, and Food Red No.105 stayed in footpads longer
Figure 2 PLN cellularity index of xanthene dyes. The left footpad of the mouse was injected with a dose of the xanthene dyes indicated. Each circle represents the PLN cellularity index of an individual mouse. Each bar indicates the mean value of the obtained PLN cellularity indices.

Figure 3 PLN cellularity index of azo dyes. The left footpad of the mouse was injected with a dose of the azo dyes indicated. See the footnotes for Fig.2.

than other dyes. Xanthene dyes bound to proteins may influence the immune reaction by acting as haptenoids. By contrast, Food Red No.106, which does not contain halogen substituents, did not increase the PLN cellularity index at the high dose of 5 mg/mouse. Xanthene dyes with halogen substituents exert a strong inhibitory effect on the in vitro prostaglandin-synthetase system, and those without halogen substituents have a weaker inhibitory effect. Xanthene dyes possess photodynamic properties. The singlet oxygen produced by the xanthene dyes may increase the PLN cellularity index through the production of hydroperoxides. Further study is needed to clarify the mechanism for the high PLN cellularity index.

Figure 3 shows the PLN cellularity index of azo dyes including Food Red No.2, Food Red No.102, Food Yellow No.4, and Food Yellow No.5. No increase in the PLN cellularity index was observed among the azo dyes studied in this experiment, and rise of the PLN cellularity index was hardly seen at the high dose (5 mg/mouse). Thus, no antigenicity of azo dyes was detected. The azo dyes seemed to migrate immediately from the footpads into the bloodstream. The immediate migration might exert the low reactivity.

The PLN cellularity index obtained with triphenylmethane dyes including Food Green No.3 and Food Blue No.1 are shown in Figure 4. There were hardly any increases in the PLN cellularity index with the two dyes, and both dyes showed the immediate disappearance from the footpads.

The PLN cellularity indexes obtained with the Indigo Carmine dye, Food Blue No.2 is shown in Figure 5. At the doses of 0.5 mg/mouse and above, Food Blue No.2 significantly increased the PLN cellularity index. The dye did not show the immediate disappearance from the footpads. At 12 days after the first injection, a small spot of Food Blue No.2 was observed in the left footpad. It is known that Food Blue No.2 as well as natural blue dye, indigo easily binds to proteins. Dye-protein complex was formed by chemical reaction of amino group on protein surface with sulfonic acid group of dye, and Food Blue No.2 gave protein binding. Therefore, Food Blue No.2 may act as a hapten. Allergy reactions associated with indigo carmine were reported.

The correlation between the structure of dyes and the reactivity seems obvious. Xanthene dyes and Indigo Carmine dye.
showed high PLN cellularity index and others did not. The dyes associated with a rise in the PLN cellularity index remained longer in the footpads. This suggests the binding of dyes to proteins. In fact, it is reported that Xanthene dyes and Indigo Carmine dye easily bind to proteins\(^{[10,11]}\). Therefore, the dye bound to proteins may induce the antigenicity.

The allergy against food colors has not been clinically reported except Food Yellow No.4\(^{[12]}\). The testing methods for the antigenicity of food colors using laboratory animals have not been established. The antigenicity tests using mice, including our study, cannot necessarily estimate the antigenicity in humans. But PLNA is proposed as a method of assessing immunotoxicity. Our results may suggest that PLNA has several advantages over conventional test methods as a means of testing the antigenicity to water-soluble materials including water-soluble food colors.

**IV. References**


マウス膝窩リンパ節測定法（popliteal lymph node assay: PLNA）による水溶性物質の
抗原性評価の試み
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キーワード：popliteal lymph node assay；PLNA；LLNA；synthetic dye；food additive；

概要

PLNAは簡便でアジュバントを併用しない抗原性試験法であるが、マウス局所リンパ節増殖試験(local lymph node assay: LLNA)では不向きとされた水溶性物質も対象としやす。近年、薬品の抗原性評価法への利用が検討されている。本研究では水溶性物質として着色料を用い、化学構造と二次応答の強さとの関係について比較検討した。8週令の雌BALB/cマウスの左後肢足踏皮下に着色料を投与した。初回投与12日後に2回目の投与を行い、その2日後、左右の膝窩リンパ節を取り出し、右側リンパ球数に対する比(cellularity index)を算出した。キサンテン系のうち構造が非常に類似した食塩赤色3号、食塩赤色104号、食塩赤色105号投与群(0.5mg/mouse)のindexは上昇した。一方、キサンテン系の食塩赤色106号、アソ系およびトリフェニルメタン系色素投与群においてindexの上昇はほとんどみられなかった。インピュラテロイド系染料の食塩青色2号の投与でindexの

上昇がみられた。以上の結果から、基本構造と反応性に明らかな関係がみられた。footpadから血中への移行が速やかな化合物は反応性が低い傾向を示したことから、血中移行への速度と抗原性との相関に興味をもった。

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