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Original Article

Immediate-type hypersensitivity to pyridoxal 5′-phosphate: Study of *in vivo* and *in vitro* cross-reactivity and identification of the antigenic determinant

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Abstract

Background: A case in which a 45-year-old female patient with peritonitis experienced immediate-type hypersensitivity on two occasions after administration of preparations containing six different medications was referred to us for closer inspection.

Methods/Results: Skin tests performed on the six medications revealed a positive reaction to the vitamin B_6 preparation Biosechs (Wakamoto Pharmaceutical, Tokyo, Japan). Further investigation showed that the principal ingredient, pyridoxal 5′-phosphate (PLP), produced a positive reaction, whereas the injection solvent with the principal ingredient removed produced a negative reaction. When compounds similar to PLP were tested, pyridoxine (PN), pyridoxamine and pyridoxal produced a negative reaction, whereas pyridoxine 5′-phosphate and pyridoxamine 5′-phosphate (PMP) produced a positive reaction. Adenosine 2′-phosphate and adenosine 5′-diphosphate were also tested and these produced a negative reaction. When a histamine-release test was performed, PLP and PMP produced a positive reaction, whereas PN produced a negative reaction. When all tests were performed on three control subjects, the results were all negative.

Conclusions: In this very rare case, phosphate radical conjugates with a pyridine nucleus became haptenic-epitope and an immediate-type reaction occurred. In past cases involving hypersensitivity to vitamin B_6, two cases involved a photoallergic reaction caused by PN and one case involved an immediate-type hypersensitivity caused by PLP. In the past cases, closely related substances had not been tested and an epitope was not identified.

Key words: cross-reactivity, haptenic epitope, histamine-release test, immediate-type hypersensitivity, skin test, vitamin B_6.

Introduction

Vitamin B_6 is a soluble vitamin that was discovered in 1934 by György[1] while investigating factors preventing dermatitis in rats. Vitamin B_6 has three forms: pyridoxine (PN), pyridoxal (PL) and pyridoxamine (PM). In the living body, vitamin B_6 is readily taken up by cells and phosphorylation occurs under Mg and Zn by action of pyridoxal kinase, producing pyridoxine 5′-phosphate (PNP), pyridoxal 5′-phosphate (PLP) and pyridoxamine 5′-phosphate (PMP). Pyridoxine 5′-phosphate oxidase causes PNP and PMP to further transform into PLP. Inside cells, the content of the phosphorylated form, predominantly PLP and PMP, far outweighs the free-form of vitamin B_6.[2] Pyridoxal 5′-phosphate and PMP work as coenzymes for amino acids and carbohydrate metabolism and play an important role in the propagation and differentiation of cells.[3] Although it is difficult to imagine how the physiologically working vitamin B_6 can become an allergen, on rare occasions there are reports of hypersensitivity occuring when vitamin B_6 was administered in a medication. In the present study, we examined a case where an immediate-type hypersensitivity occurred after

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the PLP-based Biosechs (Wakamoto Pharmaceutical, Tokyo, Japan) was administered. We also investigated cross-reactivity and possible antigenic determinants by testing PLP and similar substances *in vivo* and *in vitro*.

Case report

The patient was a 45-year-old female with no family history of atopy. In April 1993, the patient underwent surgery to remove the right ovary due to an ovarian cyst. In April 1995, the patient sought treatment at a local hospital for sudden abdominal pain. The patient was diagnosed with postoperative adhesive ileus and peritonitis. Upon admission, the patient received a saline transfusion containing Buscopan (Tanabe Pharmaceutical, Osaka, Japan) and a vitamin preparation. Directly following this, the patient experienced widespread pruritus. The transfusion was stopped and the patient's condition improved with rest and she was subsequently discharged. On 12 November 1997, the patient again sought treatment at the local hospital for abdominal pain. The patient was diagnosed with recurrent postoperative adhesive ileus and peritonitis. Upon admission, the patient received the same transfusion as on the previous occasion. Approximately 10 min into the transfusion, the patient was observed to have full body flushing, low blood pressure and blurred consciousness. The transfusion was stopped and, after a steroid was administered systemically, the patient's condition improved and she was discharged. The case was then referred to us for closer inspection.

Physical examination of the patient revealed a middle-aged woman of medium height and medium build in good nutritional condition. No organ abnormalities in the region of the thorax and abdomen were noted. No signs of erythema, wheal or edema on the skin were present.

Laboratory investigations revealed leukocytes of 8500/mm^3 (neutrophils 60.7%, eosinophils 1.2%, basophils 6.4%, lymphocytes 33.8%, monocytes 3.9%). No abnormal hepatic function was found. The serum IgE level was 161.0 IU/mL.

METHODS

Skin tests

Skin test of the administered medication

To determine which medication was the cause of the patient's reaction, a prick test (Table 1) was performed on the patient using various concentrations of each of the six medications administered during the transfusion. For medications that produced a negative reaction in the

Table 1 Reagents and results of skin testing using prescribed drugs

Reagents	Concentration	Prick test	Scratch test	Intradermal test
Buscopan (scopolamine butylbromide 20 mg/mL; Tanabe Pharmaceutical, Osaka, Japan)	As is	–	–	–
Metabolin (thiamine 50 mg/mL; Takeda Pharmaceutical, Osaka, Japan)	As is	–	–	–
Fravitan (FAD 10 mg/mL; Toa-eiyo Pharmaceutical, Tokyo, Japan)	As is	–	–	–
Biosechs (pyridoxal phospate 10 mg/mL; Wakamoto Pharmaceutical, Tokyo, Japan)	As is	+	ND	ND
	1.0 mg/mL	+	ND	ND
	0.1 mg/mL	+	ND	ND
	0.01 mg/mL	–	ND	ND
Ascoltin (ascorbic acid 100 mg/mL; Tokyo Tanabe Pharmaceutical, Tokyo, Japan)	As is	–	–	–
Keywan (phytonadione 10 mg/mL; Eizai Pharmaceutical, Tokyo, Japan)	As is	–	–	–
Normal saline	As is	–	–	–

Prick test[15] reactions were read at 15 min and a wheal larger than 5 mm mean diameter or erythema larger than 15 mm mean diameter was recorded as positive; scratch test reactions were read at 15 min and a wheal wider than 5 mm transverse diameter or erythema wider than 15 mm transverse diameter was recorded as positive; intradermal test[15] reactions were read at 15 min and a wheal larger than 9 mm mean diameter or erythema larger than 21 mm mean diameter was recorded as positive (+).

FAD, flavin adenine dinucleotide; ND, not done.

Table 2 Reagents and results of skin testing using Biosechs (Wakamoto Pharmaceutical, Tokyo, Japan) ingredient and solvent

Reagents	Concentration (mg/mL)	Prick test	Scratch test	Intradermal test
Pyridoxal 5′-phosphate	10	+	ND	ND
	1	+	ND	ND
	0.1	–	–	–
	0.01	–	–	–
Solvent*		–	–	–
Normal Saline		–	–	–

*The solvent for Biosechs excluded pyridoxal 5′-phosphate and was composed of sodium bisulfate, benzyl alcohol, hydrochloric acid and sodium hydroxide.

ND, not done.

prick test, scratch and intradermal tests were performed in that order. For control purposes, three age-matched women of normal health were administered the same tests as the patient. All tests were performed after fully informed consent had been obtained.

Skin test of Biosechs

From the above tests, the medication that caused the reaction in the patient was found to be Biosechs, the principal component of which is PLP. A skin test (Table 2) was performed using a dilution series prepared from a physiological saline solution of this PLP (Chugai Pharmaceutical, Tokyo, Japan). A skin test was also performed using the injection solvent with the PLP removed (consisting of sodium bisulfate, benzyl alcohol, hydrochloric acid and sodium hydroxide). For control purposes, three women of normal health were administered the same tests as the patient.

Skin test of compounds chemically related to PLP

Other than PLP, skin tests (Table 3; Fig. 1) were performed in the following order using PMP (Sigma Chemical Co., St Louis, MO, USA), PNP and PL-HCl (Takeda Chemical Industries, Tokyo, Japan), PN-HCl (Sigma Chemical Co.), adenosine 5′-diphosphate (Sigma Chemical Co.) and adenosine 2′-phosphate (Sigma Chemical Co.). The PNP was synthesized and supplied by Taiho Pharmaceutical (Tokyo, Japan).

Table 3 Skin testing using vitamin B_6 derivatives and related compounds

Reagents	Concentration (mg/mL)	Prick test	Scratch test	Intradermal test
PMP	1	+	ND	ND
	0.1	+	ND	ND
	0.01	–	ND	ND
PNP	1	–	–	+
	0.1	–	–	+
	0.01	–	–	+
	0.001	ND	ND	+
	0.0001	ND	ND	–
PN-HCl	1	–	–	–
	0.1	–	–	–
	0.01	–	–	–
PL-HCl	1	–	–	–
	0.1	–	–	–
	0.01	–	–	–
ADP	1	–	–	–
	0.1	–	–	–
	0.01	–	–	–
AMP	1	–	–	–
	0.1	–	–	–
	0.01	–	–	–

PMP, pyridoxamine 5′-phosphate; PNP, pyridoxine 5′-phosphate; PN-HCl, pyridoxine hydrochloride; PL-HCl, pyridoxal hydrochloride; ADP, adenosine 2′-diphosphate; AMP, adenosine 5′-phosphate.

Histamine-release test

After considering the results of the skin tests, three preparations (PLP, PMP and PN-HCl) were used as allergens in a histamine-release test (HRT) of the peripheral blood of the patient and the three control subjects. An Immunotech histamine enzyme-linked immunosorbent assay kit (Immunotech A; Beckman Coulter Company, Marseille, France) was used for this test. The test procedure followed the directions supplied with the kit. Briefly, heparinized venous blood was diluted and the allergen was added to the blood. After incubating for 30 min at 37°C, the sample was centrifuged and the supernatant

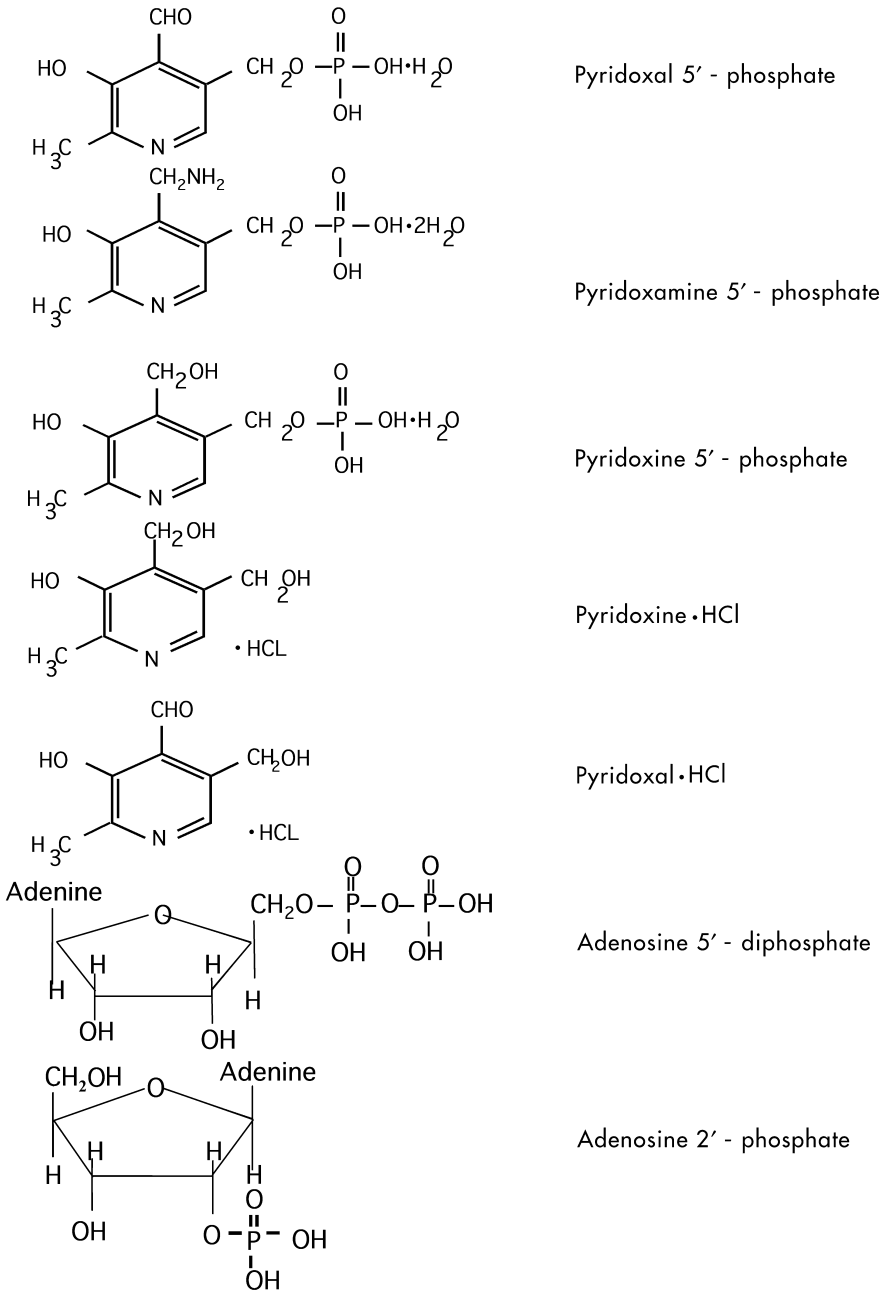

Fig. 1 Molecular structures of the chemical reagents used in the present study.

was taken as the specimen. An acylation solution was added to the specimen as well as to a series of histamine standard solutions. After agitation, this solution and enzyme-labeled acylated histamine was added to a microcup coated with anti-acylated histamine monoclonal antibody and left at 4°C for 18 h for a competitive reaction to take place. After the microcup was washed, a substrate solution (*P*-nitrophenyl phosphate) was added and the mixture was left at 23°C for 30 min. After a stop solution was added to the mixture, absorbance was measured at 410 nm and the histamine concentration was calculated. All measurements were performed in duplicate and reoccurence was confirmed on three independent experiments.

To assess total histamine, 950 μL distilled water was added to 50 μL venous blood. This solution was frozen and thawed twice and the supernatant of the lyzed cell suspension was acylated as described above. To assess

spontaneous histamine release, histamine release buffer was added instead of allergen solution and was processed in the same manner as other tubes.

Released histamine was express as a percentage and calculated as follows:

$$\%\ \text{Histamine release} = (\text{Histamine in allergen stimulation} - \text{Histamine in spontaneous release})/(\text{Total histamine} - \text{Histamine in spontaneous release}) \times 100$$

Lymphocyte stimulation tests

Lymphocyte stimulation tests (LST) were performed using standard methods in order to further investigate the case *in vitro*. For use as antigens, three dilutions of two preparations, namely PLP and PN, were prepared for testing and mononuclear leukocytes were separated from the heparinized venous blood of the patient and three healthy subjects. The antigen solution was then added to the mononuclear leukocytes. After being left to incubate at 37°C for 70 h, [^{3}H]-thymidine (18.5 MBq) was added to the mixture and it was left for an additional 2 h. A scintillation counter was used to measure the radioactivity of the [^{3}H]. A stimulation index was calculated using the following equation:

$$\text{Stimulation index}\ (\%) = (([^3\text{H}]\ \text{in the group with medication})/([^3\text{H}]\ \text{in the group without medication})) \times 100$$

The same test was administered to three healthy subjects who functioned as a control group.

Statistical analysis

The non-parametric Mann–Whitney *U*-test was used for the statistical analysis or results from the HRT and LST and $P < 0.05$ was taken as the level of significance.

RESULTS

Skin test

Skin test of administered medication

Of the six administered medications, only the vitamin B_6 preparation Biosechs produced a positive reaction during the prick tests (Fig. 2). The other medications produced a negative reaction to the prick, scratch and intradermal tests. When four dilutions of Biosechs were tested, a positive reaction to the test solution was produced in the patient by prick test until a concentration of 0.1 mg/mL solution. The three normal control participants produced negative reactions to all six medications (Table 1).

Skin test of Biosechs

In prick tests that were performed using a dilution series prepared from physiological saline and PLP (the principal component of Biosechs), a positive reaction was exhibited by the patient until a concentration of 1 mg/mL was used (Table 2). The reason for the different positive threshold compared with the results for diluted Biosechs in Table 1 is thought to be because of the dissolution of PLP in physiological saline before use resulting in a different pH of the solution and a reduced activity. The Biosechs solvent alone, with the PLP removed, produced negative reactions in the patient, even following intradermal tests. All three healthy control subjects had negative reactions to the prick, scratch and intradermal tests at a dilution of 10 mg/mL PLP.

Skin test of compounds chemically related to PLP

In tests that investigated the effects of PLP and PMP, the prick test produced positive reactions in the patient up until a dilution of 0.1 mg/mL and when a 0.01 mg/mL solution was tested, this solution produced a negative reaction. In tests that used PNP, both the prick and scratch

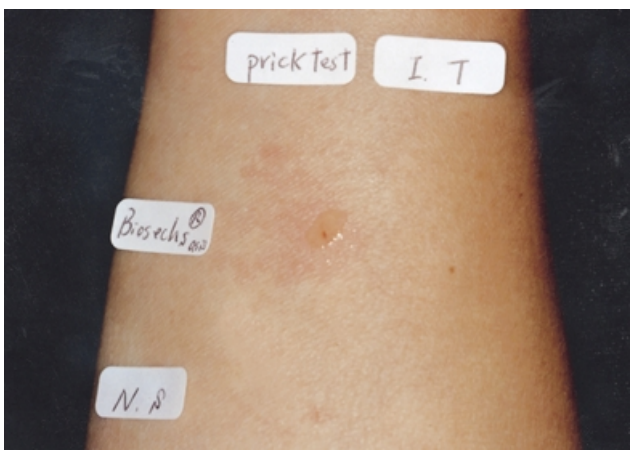

Fig. 2 Positive prick test for Biosechs (Wakamoto Pharmaceutical, Tokyo, Japan). After 1 drop of solution was applied to the forearm skin, a 27 G syringe was used to prick the skin through the solution. After 15 min, the Biosechs test had developed a wheal measuring 2 × 3 mm and erythema measuring 15 × 18 mm, whereas the physiological saline test had developed a wheal measuring 1 × 1 mm and erythema measuring 4 × 4 mm. For the three control subjects, the wheal developed to both Biosechs and physiological saline was < 2 mm in mean diameter and the erythema was < 8 mm in mean diameter. NS, normal saline.

tests produced negative reactions for all concentrations investigated. However, in the intradermal test, a positive reaction was produced in the patient with solutions up to 0.001 mg/mL (Table 3). All tests in the three control subjects produced negative reactions. The pyridine compounds without phosphate radicals (PN-HCl and PL-HCl) produced negative reactions in all tests (prick, scratch and intradermal tests) in both the patient and healthy controls. Similar negative results were observed for adenosine 5′-diphosphate and adenosine 2′-phosphate, both of which have a preserved phosphate radical but have had the pyridine nucleus replaced with another structure (Table 3).

Histamine-release test

On the basis of results of the skin tests, PLP, PMP and PN-HCl, used as antigens in the HRT, were diluted into 100, 10, 1.0 and 0.1 ng/mL solutions (Fig. 3).

When PLP was used in the HRT, the mean (±SD) % histamine release from the patient's blood with a 100 ng/mL solution was 3.93 ± 0.68, whereas the mean % histamine release for the three control participants was 0.33 ± 0.14, 1.37 ± 0.79 and 0.50 ± 0.13, indicating a significant increase in % histamine release in the patient's blood ($P < 0.02$).

When PMP was used to stimulate the blood at a concentration of 100 ng/mL, the % histamine release of the patient's blood was 3.08 ± 1.10, whereas the % histamine release of the three control participants was 0.11 ± 0.09, 1.17 ± 0.09 and 0.30 ± 0.00. The increase in histamine release from the patient's blood was highly significant ($P < 0.02$). However, when PN-HCl (which does not have phosphate radicals) was used at a concentration of 100 ng/mL, the histamine release from the patient's blood was similar to the histamine release of the three control subjects and no evidence of increased histamine release from the patient's blood was seen. The results were similar in the intradermal test. The mean spontaneous histamine release of the patient's blood in these experiments was 8.76 ± 2.44 mmol/L and the mean total histamine release was 516.93 ± 5.96 mmol/L.

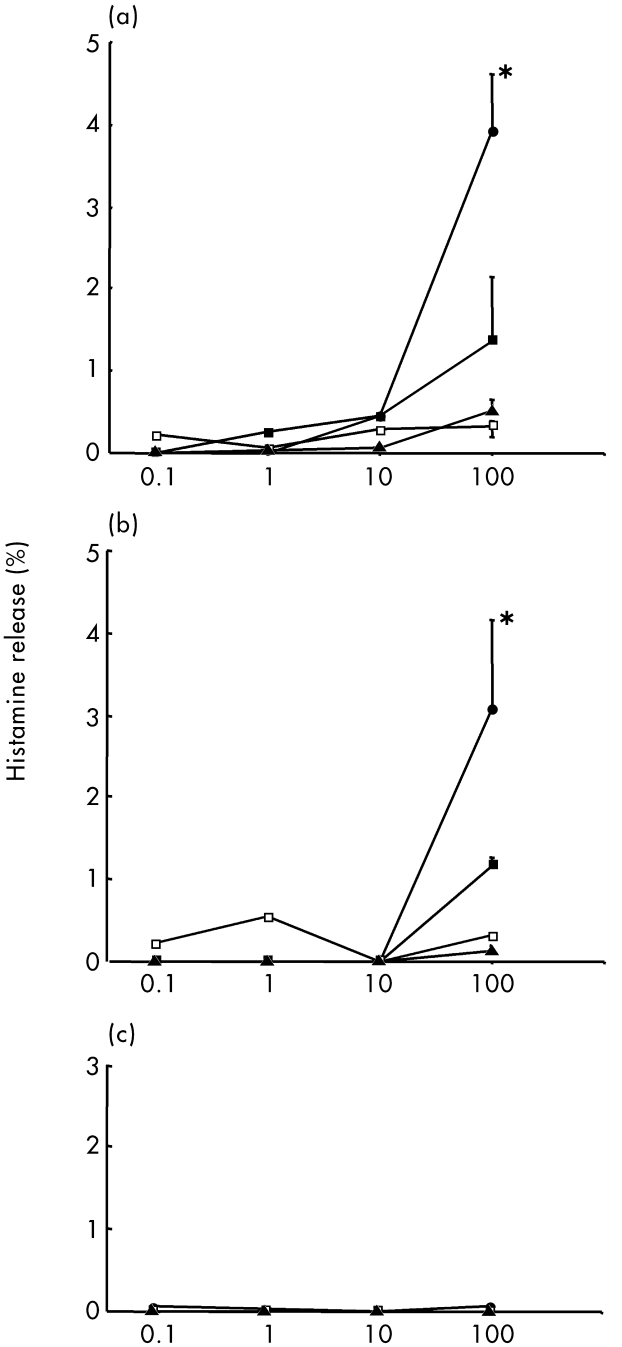

Fig. 3 Histamine release test. A significant increase (*$P < 0.02$) in histamine was observed when using (a) pyridoxal 5′-phosphate and (b) pyridoxamine 5′-phosphate. (c) No significant release of histamine was observed when using pyridoxine hydrochloride. (●), patient; (▲), control subject 1; (■), control subject 2; (□), control subject 3.

Lymphocyte stimulation tests

No increase in the stimulation index was observed in the patient's blood following stimulation with any concentration of PLP and PN-HCl. These results were similar to those obtained for the three control subjects (data not shown) and support the idea that LST is predominantly a reflection of cellular immune activity.

Discussion

Vitamin B_6 is a physiological substance that plays an important role in the metabolism of amino acids and carbohydrates inside living bodies. Pyridoxal 5′-phosphate is the active form of vitamin B_6 that occurs inside cells and it is hard to imagine of PLP itself functioning as an allergen inside a living body. Nevertheless, when administered as a medical preparation, an allergic reaction to PLP can, on rare occasions, occur and, in the past, there have been three reported cases of such an allergy occurring.[4–6] Two of these cases involved a photoallergic drug eruption caused by PN-HCl[6] or pyritinol,[5] which is a compound consisting of two PN molecules bonded by a disulfide linkage. The other case[4] was the same as the case presented herein, in which an immediate-type allergic reaction was caused by PLP and a positive reaction was acquired following a scratch test and the skin window technique using a 1% PLP solution. However, the reactions with PMP, PNP, PL and PN, and other agents, were not studied and, with regard to speculation on the mechanism of hypersensitivity, the authors of this case report pointed out the possibility of an allergic reaction caused by impurities contained in the preparation or the possibility of patient's mast cells becoming hypersensitive to PLP in a-non-allergic way.[4]

Contrary to the findings of the previous case, we have shown, by performing skin reaction tests and HRT, that our patient has hypersensitivity and that there was a reaction to the principal agent of the injection administered to the patient, but not a reaction to the solvent. There was a reaction to PLP, PMP and PNP, but no reaction to PL and PN. Furthermore, there was no reaction to adenosine 5′-diphosphate and adenosine 2′-phosphate, similar compounds that contain phosphate side chains. From this, we speculate that it could be an allergic reaction that is only valid with PLP, PMP and PNP, which have a pyridine nucleus and also phosphate radicals attached. This raises the question of how these compounds, which are metabolites of vitamin B_6 inside the cells,[2] have acquired antigenic properties.

The molecular basis of antigen presentation in drug allergy has not been fully elucidated. In general, immature dendritic cells in the peripheral tissue take up exogenous antigens as antigen-presenting cells (APC) through phagocytosis and macropinocytosis. The internalized antigen gets broken down into peptide fragments in acidic organelles, such as endosomes and lysosomes, by protease and is combined with major histocompatibility complex (MHC) class II molecules that can then be observed on the cell surface. However, recently class I molecules have also been observed to be capable of presenting foreign antigens.[8,9] Moreover, CD1 molecules have also been found to be capable of being antigen-presenting elements.[10] Following antigen presentation, T cell receptor (TCR) signaling is the next decisive factor in the initiation of a specific immune reaction. The T cell response, which includes Th1/Th2 polarization, is affected by various types of antigens, doses, costimulatory factors and cytokine signals that are accessible to antigen-specific T cells.

Allergy to medication is analogous to other chemical haptens in that the ability of any drug to induce an immune reaction depends on its reactivity with proteins. The molecular targets of such a haptenation process may be soluble or cell-bound proteins, including MHC molecules and associated peptides.

The nature of the hapten determinant has only recently started to be investigated. In the case of penicillin allergy,[11] the antigenic determinant contacted by the specific TCR is formed by the contribution of both the side chain and backbone structures of the penicillin molecule. The penicillin-specific T cell response is MHC restricted, with a strong predominance of human leukocyte antigen-DR restriction for CD4+ T cell clones.[12] Penicillin can also be presented to specific T cells by glutaraldehyde-fixed autologous B cells, implying the covalent formation of antigenic epitopes without the requirement of intracellar processing.[13] Concerning metal allergy,[14] metals, via their chemically reactive groups, could attach themselves to peptides bound to MHC molecules and induce MHC-restricted responses to the conjugates. The metals themselves may not necessarily have been internalized by APC.

When we consider vitamin B_6, it is hard to imagine that the APC is including it as a foreign substance, even when PL and PLP, which are intrinsic physiological substances, are administered as medication in large doses. However, just like penicillin allergy and metal allergy, we cannot rule out the possibility of vitamin B_6 combining with peptides residing in either the MHC grooves of APC or B cells or with CD1 molecules and being recognized by TCR and turning on signals. Vitamin B_6 found in the diet exists in minute quantites (0.01 mg/g in chicken) and vitamin B_6 levels in plasma are 20–30 mmol/L (0.0053–0.0079 mg/mL), which are far below the threshold of the hypersensitivity reaction. Pyridoxal 5′-phosphate, PMP and PNP, which occurs in

comparatively larger quantities, are inside cells. Because of this, they have no opportunity to come into contact with MHC. Therefore, even after sensitization has been established, an immune reaction does not occur. From this, one can understand why hypersensitivity to vitamin B_6 occurs only rarely and a reaction only occurs when vitamin B_6 is administered as a medication, even with an established sensitization.

Physiological substances such as vitamins are often treated as substances that are safe to be administered as medications. However, when large dosages are administered intravenously all at once, the patient should be monitored for the occurence of hypersensitivity.

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