

Effect of Cobalamin on the Allergic Response in Mice

Ulala FUNADA,* Masahiro WADA,**,[†] Tetsunori KAWATA,*** Nobuo TANAKA,****
Tadahiro TADOKORO,* and Akio MAEKAWA*

*Department of Applied Biology and Chemistry, Faculty of Applied Bioscience, Tokyo University of Agriculture, Setagaya-ku, Tokyo 156-8502, Japan

**Department of Nutritional Science, Faculty of Applied Bioscience, Tokyo University of Agriculture, Setagaya-ku, Tokyo 156-8502, Japan

***Faculty of Education, Okayama University, Tsushimanaka, Okayama 700-0082, Japan

****Seifuso Hospital, Niiza, Saitama 352-0023, Japan

Received March 3, 2000; Accepted May 16, 2000

Cyanocobalamin, deoxyadenosylcobalamin and methylcobalamin were separately administered to mice sensitized with the ovalbumin antigen to investigate the allergic response. The serum IgE and pulmonary histamine concentrations were significantly lower in all cobalamin (Cbl)-administered groups. The production of interleukin (IL)-2 and IL-4 in splenocytes was also lower in all Cbl-administered groups. These results show that Cbls were effective in mitigating allergic reactions and IL-2 production. CD3⁺CD28⁺ (CD28 is an accessory molecule related to IL-2 production) and CD4⁺CD28⁺ in splenocytes were higher in all the Cbl-administered groups. However, CD3⁺CD28⁻, CD4⁺CD28⁻ and CD5⁺CD25⁻ (CD25: IL-2 R α /p55) were lower in the Cbl-administered groups. In addition, Cbl specifically inhibited the cellular phosphorylation of tyrosine induced by ovalbumin sensitization. These results indicate that the signal in a cell by CD 28 was restrained by Cbl. We infer that Cbl administration significantly reduced the IL-2 concentration, and secondarily the IL-4, IgE and histamine concentrations.

Key words: cobalamin; allergy; IgE; IL-2; IL-4

Four kinds of cobalamin (Cbl) analogues, cyanocobalamin (CN-Cbl), hydroxycobalamin (OH-Cbl), deoxyadenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl), are currently known. The main forms of *in vivo* Cbl in humans are considered to be AdoCbl and MeCbl. AdoCbl acts as a coenzyme of methylmalonyl CoA mutase [EC 5.4.99.2] and MeCbl as a coenzyme of methyltetrahydrofolate-methyltransferase [EC 2.1.1.13] in the metabolism of proteins, nucleic acids, fats and carbohydrates.

These facts suggest that cell proliferation may be altered by AdoCbl and MeCbl. While CN-Cbl and OH-Cbl are scarcely found in the human body, when administered orally or intradermally, they are each transformed into active Cbl in the liver within several days, combined with transcobalaminII, and enter the cells *via* the receptor.¹⁾ On the other hand, when a large amount of Cbl is administered, a portion is directly absorbed by diffusion without involving in the intrinsic factor.²⁾ However, there have been few studies about the absorption of Cbl analogues,³⁾ so their significance remains largely unknown.

Cbl deficiency caused by insufficient ingestion or by Cbl malabsorption is known to cause various disorders in mammals. Typical diseases caused by Cbl deficiency are pernicious anemia^{4,5)} and peripheral neuropathy,⁵⁾ and immunodeficiency in pernicious anemia, *i.e.*, a change in T cells, has been clinically observed.^{6–11)} We have already reported that Cbl deficiency lowered the serum C3 concentration in rats, which could be recovered by MeCbl administration, and thus concluded that MeCbl participated in regulating the complementary function.¹²⁾

On the other hand, Sakane *et al.*¹³⁾ have examined the immune function under Cbl-sufficient conditions and found that MeCbl promoted the function of helper T cells *in vitro*. They also suggested that MeCbl promoted the production of concanavalin A-induced suppressor cells, and thus explained that MeCbl improved not only an immunodeficiency such as that observed in pernicious anemia, but also the general immunodeficiency mediated by the T-cell function.

We examined cytokine production and especially IL-2 production. IL-2 is a factor of T cell prolifera-

[†] To whom correspondence should be addressed: Tel: 81-3-5477-2458; Fax: 81-3-5477-2626; E-mail: mwada@nodai.ac.jp

Abbreviations: Cbl, cobalamin; CN-Cbl, cyanocobalamin; OH-Cbl, hydroxycobalamin; AdoCbl, deoxyadenosylcobalamin; MeCbl, methylcobalamin; IL, interleukin; TCR, T cell receptor; pTh, naive T cells; OVA, ovalbumin; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; R-PE, R-Phycoerythrin

tion, and the antigen must combine with the T cell receptor (TCR)/CD3 complex in the activation of naive T cells (pTh), while secondary signals mediating CD28 are also required. CD28 is an accessory molecule related with the activation of pTh, and the stimulation of pTh induces phosphorylation of cellular proteins, transmits signals to the nucleus, and induces IL-2 production. In addition, CD25 (IL-2 R α /p55) was also examined.

We administered AdoCbl or MeCbl to mice in the active coenzyme form, or CN-Cbl in the inactive coenzyme form,¹⁴ the mice having been sensitized with the ovalbumin (OVA) antigen. We examined the humoral, cell-mediated immune functions and cytokine levels in the mice from the viewpoint of the allergic reaction.

Materials and Methods

Animals and diet. Six-week-old male BALB/c mice (Tokyo Experimental Animals Science Co. Ltd., Tokyo, Japan) were used and assigned to groups of seven animals. The diet used for the experiment was prepared according to Table 1. In order to administer Cbl by intraperitoneal injection (i.p.), we fed the animals on a Cbl-free diet for the experimental period (12 days). The animals had free access to the diet and water. The conduct of the animal experiment followed "The standard on breeding and storage of the laboratory animal" (The Prime

Minister's Office announcement No. 6 in March, 1980).

Sensitization and Cbl administration. OVA, CN-Cbl, AdoCbl and MeCbl were purchased from Sigma Chemical Co. (St. Louis, U.S.A.), and *Bordetella pertussis* was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The mice were sensitized by adding 5 mg of OVA to 1 ml of a solution containing 0.9% (w/v) NaCl and 9% (w/v) potassium aluminum hydroxide adjusted to pH 6.5. Then, 1×10^{11} inactivated *Bordetella pertussis* was added as an adjuvant, and 0.1 ml per animal of the mixture was i.p. administered to the mice. Eleven days later, 0.1 mg/kg body wt. of CN-Cbl, AdoCbl, MeCbl or physiological saline (control) was i.p. administered once. Blood was collected 24 hours later from the heart under nembutal anesthesia and centrifuged at $1,300 \times g$, the serum being separated and stored at -30°C until needed. The liver, spleen and lung were removed from the mice, the liver being stored at -30°C until needed.

Hepatic Cbl concentration. The hepatic Cbl concentration was determined by a microbiological assay using *Lactobacillus delbruekii lactis* ATCC 7830. Cbl was extracted from rat liver by boiling in the presence of KCN, essentially as described by Frenkel *et al.*¹⁵⁾

Serum C3, IgG, IgE and pulmonary histamine concentrations. The concentration of C3 was measured by a single radial immunodiffusion assay,¹⁶⁾ and IgG and IgE in the serum were measured by an enzyme-linked immunosorbent assay¹⁷⁾ (ELISA). The pulmonary histamine concentration was measured by the fluorescence method of May *et al.*¹⁸⁾

Cytokine level in splenocytes. Immediately after its isolation, the spleen was perfused with phosphate-buffered saline (pH 7.2), the perfusate was collected, and a cell suspension was obtained. The cell suspension, after being loaded on Lympholyte-M (Cedarlane Laboratories Ltd., Ontario, Canada), was centrifuged at $500 \times g$ for 20 min at room temperature, and splenocytes were obtained from the middle layer. The splenocytes of 5×10^5 cells was incubated in 1 ml of a RPMI 1640 medium (Wako) containing 10% fetal calf serum at 37°C in 5% CO_2 for 48 hours in the presence of the anti-mouse CD3 (Caltag Laboratories, Burlingame, Canada) and CD28 (Caltag) antibodies. The supernatant of the culture was quantified with an Inter Test-2X Mouse IL-2 ELISA kit (Genzyme Co., Cambridge, U.S.A.) and Inter Test-4X Mouse IL-4 ELISA kit (Genzyme).

Proportions of CD3, CD4, CD5, CD25 and CD28 in the splenocytes. The splenocytes were treated with anti-mouse monoclonal antibodies to CD3 that had

Table 1. Composition of the Experimental Diet

Ingredient	g/kg of diet
Soybean protein ¹	180.0
Glucose anhydrous ²	673.5
Lard ³ containing fat-soluble vitamins ⁴	100.0
Mineral mixture ⁵	35.0
Water-soluble vitamin mixture, B12-free ⁶	10.0
Choline chloride ⁷	1.5

¹ Ajipron SU, 85.4% crude protein content Ajinomoto Co., Tokyo, Japan.

² Sanei Toka Co., Ehime, Japan.

³ Hayashi Chemicals Co., Tokyo, Japan.

⁴ Fat-soluble vitamins in 1 kg of diet: alpha-tocopheryl acetate, 35 mg; retinyl acetate, 4,000IU; and cholecalciferol, 1,000IU. These vitamins were purchased from Wako Pure Chemical Industries, Osaka, Japan.

⁵ Mineral mixture contained the following (g/kg of mixture): calcium phosphate, dibasic, 500; sodium chloride, 74; potassium citrate, monohydrate, 220; potassium sulfate, 52; magnesium oxide, 24; manganous carbonate, 3.5; ferric citrate, 6; zinc carbonate, 1.6; cupric carbonate, 0.3; potassium iodate, 0.01; sodium selenite, 0.01; and chromium potassium sulfate, 0.55. Weight of the mixture was made up to 1 kg by the addition of glucose anhydrous. All mineral salts were purchased from Wako Pure Chemical Industries, Osaka, Japan.

⁶ Water-soluble vitamin mixture contained the following (mg/kg of mixture): thiamin hydrochloride, 600; riboflavin, 600; pyridoxine hydrochloride, 700; nicotinic acid, 3,000; calcium pantothenate, 1,600; folic acid, 200; biotin, 20; and menadione, 5. Weight of the mixture was made up to 1 kg by the addition of glucose anhydrous. All mineral salts were purchased from Wako Pure Chemical Industries, Osaka, Japan.

⁷ Wako Pure Chemical Industries, Osaka, Japan.

Table 2. Cbl Concentration in the Liver, C3, IgG and IgE Concentrations in the Serum, and Histamine Concentration in the Lungs of Mice Administered with CN-Cbl, AdoCbl and MeCbl^{1,2}

	Cbl (ng/tissue)	C3 (%)	IgG (mg/ml)	IgE (ng/ml)	Histamine (ng/tissue)
Control	118 ± 12 ^c	100 ± 3	1.41 ± 0.09	497 ± 28 ^a	314 ± 14 ^a
CN-Cbl	172 ± 10 ^b	101 ± 4	1.31 ± 0.08	342 ± 40 ^b	177 ± 8 ^c
AdoCbl	235 ± 9 ^a	109 ± 3	1.22 ± 0.05	339 ± 20 ^b	220 ± 2 ^b
MeCbl	249 ± 5 ^a	100 ± 3	1.29 ± 0.05	383 ± 17 ^b	198 ± 7 ^{bc}

¹ Each value is the mean ± SEM for seven mice.² Values with different superscript letters are significantly different ($p < 0.05$).

been labeled with fluorescein isothiocyanate (FITC, Caltag), to CD4 that had been labeled with FITC (Serotec Ltd., Oxford, U.K.), to CD5 that had been labeled with R-Phycoerythrin (R-PE, Serotec), to CD25 that had been labeled with FITC (Cedarlane) and to CD28 that had been labeled with R-PE (Caltag). Splenocyte subsets of the obtained 1×10^6 cell groups were gated and measured with a flow cytometer (Epics Elite; Coulter, U.S.A.).

Analysis of phosphotyrosine in the splenocytes.

After dissolving the splenocytes, they were subjected to SDS-PAGE,¹⁹ and phosphotyrosine was detected by a western blotting analysis,²⁰ using an anti-phosphotyrosine antibody (Upstate Biotechnology, NY, U.S.A.).

Statistical analysis. The significance of intergroup difference was tested by one-way ANOVA with SPSS, and then by a parametric analysis, Duncan's multiple range test being performed at $p < 0.05$. Heteroscedasticity was evaluated with the Kruskal-Wallis test as a non-parametric analysis at $p < 0.05$.

Results

Hepatic Cbl, serum C3, IgG, IgE and pulmonary histamine concentrations

The hepatic Cbl concentration was significantly higher ($p < 0.05$) in the CN-Cbl, AdoCbl and MeCbl groups than in the control group (Table 2). The serum C3 and IgG concentrations were no different among the groups. On the other hand, the serum IgE concentration was significantly lower ($p < 0.05$) in the CN-Cbl, AdoCbl and MeCbl groups than in the control group (Table 2). At though the IgG and IgE concentrations were not measured as OVA specific antibodies, there was no difference in the IgG concentration after OVA sensitization (control, 1.41 ± 0.09 mg/ml; CN-Cbl, 1.31 ± 0.08 mg/ml; AdoCbl, 1.22 ± 0.05 mg/ml; MeCbl, 1.29 ± 0.05 mg/ml) than that before (control, 2.07 ± 0.06 mg/ml; CN-Cbl, 1.58 ± 0.13 mg/ml; AdoCbl, 1.50 ± 0.08 mg/ml; MeCbl, 1.52 ± 0.06 mg/ml). However the IgE concentration after OVA sensitization (control, 497 ± 28 ng/ml; CN-Cbl, 342 ± 40 ng/ml; AdoCbl, 339 ± 20 ng/ml;

Table 3. Cytokine Content in the Splenocytes of Mice Administered with CN-Cbl, AdoCbl and MeCbl^{1,2}

	IL-2 (ng/ml)	IL-4 (pg/ml)
Control	3.89 ± 0.45 ^a	270 ± 31 ^a
CN-Cbl	3.37 ± 0.50 ^{ab}	146 ± 36 ^b
AdoCbl	2.37 ± 0.41 ^b	106 ± 31 ^b
MeCbl	2.53 ± 0.25 ^b	91 ± 28 ^b

¹ Each value is the mean ± SEM for seven mice.² Values with different superscript letters are significantly different ($p < 0.05$).

MeCbl, 383 ± 17 ng/ml) was higher than that before (control, 14.2 ± 2.6 ng/ml; CN-Cbl, 15.2 ± 2.6 ng/ml; AdoCbl, 15.4 ± 3.8 ng/ml; MeCbl, 11.1 ± 2.5 ng/ml). The histamine concentration was significantly lower ($p < 0.05$) in the CN-Cbl, AdoCbl and MeCbl groups than in the control group (Table 2).

Cytokine levels in the splenocytes

The IL-2 production in splenocytes tended to be lower in the CN-Cbl group than in the control group, and was significantly lower ($p < 0.05$) in the AdoCbl group and the MeCbl group (Table 3). The IL-4 production in the splenocytes was significantly lower ($p < 0.05$) in all experimental groups than in the control group (Table 3).

Proportions of CD3, CD4, CD5, CD25 and CD28 in the splenocytes

The proportion of CD3⁺CD28⁻ cells tended to be lower in the CN-Cbl group, and was significantly lower ($p < 0.05$) in the AdoCbl and MeCbl groups than in the control group. The proportion of CD3⁺CD28⁺ cells was significantly higher ($p < 0.05$) in the CN-Cbl, AdoCbl and MeCbl groups than in the control group (Table 4). The proportion of CD4⁺CD28⁻ cells tended to be lower in the CN-Cbl group and AdoCbl group than in the control group, and was significantly lower ($p < 0.05$) in the MeCbl group. The proportion of CD4⁺CD28⁺ cells tended to be higher in the AdoCbl group, and was significantly higher ($p < 0.05$) in the CN-Cbl and MeCbl groups than in the control group (Table 4). The proportion of CD5⁺CD25⁻ cells tended to be lower in the AdoCbl group than in the control group, and

Table 4. Proportions of CD3, CD4, CD5, CD25 and CD28 in the Splenocytes of Mice Administered with CN-Cbl, AdoCbl and MeCbl^{1,2}

	CD3 ⁺ CD28 ⁻ (%)	CD3 ⁺ CD28 ⁺ (%)	CD4 ⁺ CD28 ⁻ (%)	CD4 ⁺ CD28 ⁺ (%)	CD5 ⁺ CD25 ⁻ (%)	CD5 ⁻ CD25 ⁺ (%)	CD5 ⁺ CD25 ⁺ (%)
Control	40.8 ± 2.7 ^a	1.52 ± 0.27 ^b	22.3 ± 0.9 ^a	0.92 ± 0.10 ^b	33.8 ± 0.7 ^a	1.85 ± 0.24	2.37 ± 0.17
CN-Cbl	35.4 ± 2.5 ^{ab}	3.26 ± 0.28 ^a	21.5 ± 2.4 ^{ab}	1.57 ± 0.19 ^a	27.8 ± 1.6 ^b	2.34 ± 0.31	3.25 ± 0.24
AdoCbl	33.1 ± 1.1 ^b	2.73 ± 0.20 ^a	17.1 ± 1.0 ^{ab}	1.23 ± 0.08 ^{ab}	31.9 ± 0.9 ^{ab}	2.44 ± 0.12	2.72 ± 0.11
MeCbl	33.5 ± 1.7 ^b	2.68 ± 0.19 ^a	15.5 ± 0.6 ^b	1.42 ± 0.09 ^a	29.1 ± 1.6 ^b	2.10 ± 0.16	3.70 ± 0.62

¹ Each value is the mean ± SEM for seven mice.² Values with different superscript letters are significantly different ($p < 0.05$).

was significantly lower ($p < 0.05$) in the CN-Cbl group and MeCbl group. The proportions of CD5⁻CD25⁺ and CD5⁺CD25⁺ cells were no different among the groups (Table 4).

Analysis of phosphotyrosine in the splenocytes

The splenocytes in the control group showed two bands at 48 kDa and 14 kDa (arrow) for phosphotyrosine which were not present in the Cbl-administered groups. This indicates that Cbl inhibited the intracellular tyrosine phosphorylation induced by anti-mouse CD3 and CD28 antibody stimulation (Fig. 1).

Discussion

Only a few studies have been performed on the effect of Cbl on the immune function in animals without Cbl deficiency.^{13,21,22} Sakane *et al.*²² have reported that the mixed lymphocyte reaction in an autologous mixed lymphocyte culture from rheumatics was increased by the presence of MeCbl, before recovering to the normal level. They have suggested that MeCbl regulated the immune function *in vivo* by increasing the function of T cells, and that this was possibly useful to improve malfunctions of the immune systems.¹³ Recent studies have reported the efficacy of MeCbl on the immune control system.²³⁻²⁵

Accordingly, the OVA antigen was used to sensitize mice, to which CN-Cbl in the inactive coenzyme form, or AdoCbl or MeCbl in the active coenzyme form¹⁴ was then administered, and the immune function was examined in relation to allergic reaction. The Cbl dose followed the report of Glass *et al.*,²⁶ as the quantity which could be expected to provide a pharmaceutical effect.

In the groups administered with CN-Cbl, AdoCbl or MeCbl, the hepatic Cbl concentration was significantly higher than that in the control group. However, the CN-Cbl concentration was lower than the AdoCbl or MeCbl concentration, so it seems there was a different conversion and utilization of CN-Cbl in the liver.

At though the IgG and IgE concentrations were not measured as OVA specific antibodies, the IgE concentration after OVA sensitization was higher than that before, indicating that OVA sensitization

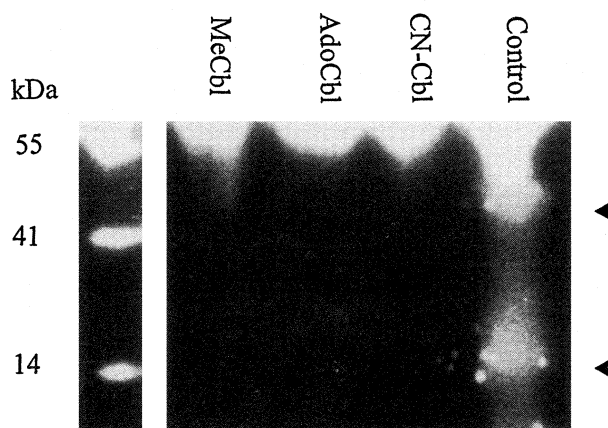


Fig. 1. Effect of Cbl on the Splenocytes of OVA Sensitized Mice. The splenocytes in the control group showed bands at 48 kDa and 14 kDa (arrow) for phosphotyrosine which were not found in the CN-Cbl, AdoCbl and MeCbl-administered groups.

had influence. The IgE and pulmonary histamine concentrations were increased by OVA sensitization, but were significantly lower than those in the control group with Cbl administration. IL-4 production in the splenocytes, an allergy-reaction factor,²⁷ was also significantly lower in all Cbl-administered groups than in the control group. These results indicate that not only MeCbl but also CN-Cbl and AdoCbl may have mitigated the allergic reactions.

However, IL-2 production in the splenocytes was also lower in all the Cbl-administered groups than in the control group. Yamashiki *et al.*²⁸ have indicated that MeCbl mainly suppressed the cytokine production of T lymphocytes *in vitro*. We infer that Cbl administration significantly reduced IL-2, and secondarily IL-4, IgE and histamine. CD4-positive cells are classified into the following three types, according to the pattern of cytokine production: Th1-type cells, Th2-type cells, and their progenitor cells Th0.²⁹⁻³¹ PTh is differentiated to Th0 cells by antigenic stimulation in the presence of IL-2.^{31,32} If the IL-2 production of pTh cells were to decrease, IL-4 production by Th2 cells may be restrained.

CD28 is an accessory molecule related to the activation of pTh, and the stimulation of pTh induces phosphorylation of cellular proteins, transmits signals to the nucleus, acts on the promoter/enhancer region of the IL-2 gene, and strongly induces IL-2

production.³³⁾ Both the signal mediated by TCR and the accessory signal mediated by CD28 are generally required for the activation of pTh, while pTh becomes the anergy only in the signal of TCR.^{34,35)}

In the present study, CN-Cbl, AdoCbl and MeCbl are considered to have caused T cells to become anergy through suppression of the CD28-mediated secondary signal. Increases in CD3⁺CD28⁺ and CD4⁺CD28⁺ were also found. However, there is the possibility that Cbl acted on pTh, considering the decrease in the two antagonizing cytokines,³⁶⁾ IL-2 and IL-4, and the decrease in the expression rates of CD3⁺CD28⁻, CD4⁺CD28⁻ and CD5⁺CD25⁻. In addition, Cbl specifically inhibited the phosphorylation of tyrosine induced by OVA sensitization.

We found that Cbls were effective in mitigating allergic reactions and IL-2 production. In addition, Cbl specifically inhibited the cellular phosphorylation of tyrosine induced by OVA sensitization. These results indicate that the signal in a cell mediated by CD 28 was restrained by Cbl. An analysis of various cells and the effect of Cbl on intracellular signal transmission should be conducted in a future study.

References

- Allen, R. H., The plasma transport of vitamin B12. *Brit. J. Haematol.*, **33**, 161 (1976).
- Okuda, K., Vitamin B12 absorption in rats studied by a "loop" technique. *Am. J. Physiol.*, **199**, 84 (1960).
- Shinton, N. K. and Troughton, O., Therapeutic response to large oral doses of cobalamin. The cobalamins. Churhill Livingstone, Edinburgh, p. 183 (1971).
- Bethell, F. H., The relation of therapy in pernicious anemia to change in the nervous system. *Blood*, **3**, 57 (1948).
- Gräsbeck, R. and Salonen, E., Vitamin B12 Prog. *Fd. Nutr. Sci.*, **2**, 193-231 (1976).
- Imamura, N., Fujiwara, K., and Kuramoto, A., Lymphocyte subpopulations in pernicious anemia. *N. Engl. J. Med.*, **311**, 56 (1984).
- Wodzinski, M. A., Forrest, M. J., Barnett, D., and Lawrence, A. C. K., Lymphocyte subpopulations in patients with hydroxocobalamin responsive megaloblastic anemia. *J. Clin. Pathol.*, **38**, 582-584 (1985).
- Gogos, C. A., Kapatais-Zoumbos, K. N., and Zoumbos, N. C., Lymphocyte subpopulations in megaloblastic anemia due to vitamin B-12 deficiency. *Scand. J. Haematol.*, **37**, 316-318 (1986).
- Kubota, K., Arai, T., Tamura, J., Shirakura, T., and Morita, T., Restoration of decreased suppressor cells by vitamin B12 therapy in a patient with pernicious anemia. *Am. J. Hematol.*, **24**, 221-223 (1987).
- Kubota, K., Kurabayashi, H., Kawada, E., Okamoto, K., and Shirakura, T., Restoration of abnormally high CD4/CD8 ratio and low natural killer cell activity by vitamin B12 therapy in a patient with post-gastrectomy megaloblastic anemia. *Internal Medicine*, **31**, 125-126 (1992).
- Tamura, J., Kubota, K., Murakami, H., Sawamura, M., Matsushima, T., Tamura, T., Saitoh, T., Kurabayashi, H., and Naruse, T., Immunomodulation by vitamin B12: augmentation of CD8⁺ T lymphocytes and natural killer (NK) cell activity in vitamin B12-deficient patients by methyl-B12 treatment. *Clin. Exp. Immunol.*, **116**, 28-32 (1999).
- Wada, M., Kawata, T., Yamada, K., Funada, U., Kuwamori, M., Endo, M., Tanaka, N., Tadokoro, T., and Maekawa, A., Serum C3 content in vitamin B12-deficient rats. *Internat. J. Nutr. Res.*, **68**, 94-97 (1998).
- Sakane, T., Takada, S., Kotani, H., and Tsunematsu, T., Effect of methyl-B12 on the *in vitro* immune functions of human T lymphocytes. *J. Clin. Immunol.*, **2**, 101-109 (1982).
- Uchino, H., Methyl B12 (methylcobalamin): clinical applications. *The Pharmaceuticals Monthly* (in Japanese), **12**, 1525-1532 (1970).
- Frenkel, E. P., Prough, R., and Kitchens, R. L., Measurement of tissue vitamin B12 by radioisotopic competitive inhibition assay and a quantitation of tissue cobalamin fractions. *Methods Enzymol.*, **67**, 31-40 (1980).
- Mancini, G., Carbonara, A. O., and Heremans, J. F., Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochem.*, **2**, 235-254 (1965).
- Bird, P., Calvert, J. E., Lowe, J., Duggan-Keen, M., Forouhi, N. G., Seppala, and Ling, N. R., ELISA measurement of IgG subclass production in culture supernatants using monoclonal antibodies. *J. Immunol. Methods*, **23**, 149-158 (1987).
- May, C. D., Lyman, M. Alberto, R., and Cheng, J., Procedures for immunochemical study of histamine release from leukocytes with small volume of blood. *J. Allergy*, **46**, 12-20 (1970).
- Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685 (1970).
- Towbin H., Staehelin, T., and Gordon, J., Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 4350-4354 (1979).
- Takimoto, G., Yoshimatsu, K., Isomura, J., Ikeda, T., Morimoto, T., and Osawa, T., Immunomodulating activity of methyl-B12. *J. Clin. Exp. Med.* (Igakuno Ayumi in Japanese), **112**, 588-590 (1980).
- Sakane, T., Murakawa, Y., Takeno, M., Shigeki, T., Nagafuchi, H., and Miki, T., T cell interactions in active rheumatoid arthritis: insights from the human autologous mixed lymphocyte reaction as a model of T cell activation cascade. *Clin. Exp. Immunol.*, **85**, 55-60 (1991).
- Ikuno, H., Yoshioka, K., and Yoshinaga, T., A clinical experience of i.m. injection of methyl B12 in patients with rheumatoid arthritis. *J. Kyushu Rheumatism Association* (in Japanese), **5**, 145-150 (1986).
- Ueo, T., Okumura, H., and Yamamuro, T., Efficacy of continuous i.m. injection of methyl B12 in patients with rheumatoid arthritis. *Clinical Immunology* (in Japanese), **19**, 901-908 (1987).
- Kawai, K. and Hirohata, K., A clinical experience of

- large oral dose administration of methyl B12 in patients with rheumatoid arthritis. *Clinical Rheumatism* (in Japanese), **2**, 143–149 (1989).
- 26) Glass, G. B. J., Applicability of hydroxocobalamin as a long-acting vitamin B12. *Nature*, **189**, 138–140 (1961).
- 27) Paul, W. E. and Ohara, J., B cell stimulatory factor-1 /interleukin 4. *Annu. Rev. Immunol.*, **5**, 429–459 (1987).
- 28) Yamashiki, M., Nishimura, A., and Kosaka, Y., Effects of methylcobalamin (vitamin B12) on *in vitro* cytokine production of peripheral blood mononuclear cells. *J. Clin. Lab. Immunol.*, **37**, 173–182 (1992).
- 29) Mosmann, T. R. and Coffman, R. L., TH1 and TH2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.*, **7**, 145–173 (1989).
- 30) Mosmann, T. R. and Sad, S., The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol. Today*, **17**, 138–146 (1996).
- 31) Paul, W. E. and Seder, R. A., Lymphocyte responses and cytokines. *Cell*, **76**, 241–251 (1994).
- 32) Street, N. E., Schumacher, J. T., Fong, T. A., Bass, H., Fiorentino, D. F., Leverah, J. A., and Mosmann, T. R., Heterogeneity of mouse helper T cells. Evidence from bulk cultures and limiting dilution cloning for precursors of Th1 and Th2 cells. *J. Immunol.*, **144**, 1629–1639 (1990).
- 33) Fraser, J. D., Irving, B. A., Grabtree, G. R., and Weiss, A., Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. *Science*, **251**, 313–316 (1991).
- 34) Linsley, P. S., Brady, W., Grosmaire, L., Aruffo, A., and Damle, N. K., Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *J. Exp. Med.*, **173**, 721–730 (1991).
- 35) Schwartz, R. H., Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. *Cell*, **71**, 1065–1068 (1992).
- 36) Trinchieri, G., Interleukin 12 and its role in the generation of Th1 cells. *Immunol. Today*, **14**, 335–338 (1993).