Immunological Characterization and Clinical Implication of Cobalamin Binding Protein in Human Gastric Cancer

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ABSTRACT

Cobalamin (vitamin B12) binding protein was purified from gastric cancer extracts and from serum-free culture medium of cancer cell line KATO-III. The molecular weight, determined by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was 70,000 and the pl was 2.8 to 3.2. From biochemical and immunological properties, this cobalamin binding protein was considered to be an isoprotein of cobalamin R binder. Monoclonal antibodies were produced against saliva R and cobalamin binding protein in culture medium to study their antigenic determinants. Monoclonal antibody 55-D reacted to an epitope of peptide in both binders, whereas WK-1 and H-12 reacted to determinants of a carbohydrate moiety, including sialic acid, in cancer cell-derived binder. In addition, we carried out an enzyme-linked immunosassay and examined plasma levels of immunoactive R binder in patients with gastric cancer (n = 72), benign gastrointestinal disease (n = 30), and healthy individuals (n = 40). Even in patients without liver metastasis, the level of immunoactive R binder detected by monoclonal antibody H-12 was elevated in some patients and decreased after excision of the tumor. R binder was also elevated in cancer tissue extract. Immunoactive binder was histochemically detected in the cytosol of cancer cells and metastatic cells of the gastric mucosa. The present findings suggest that cobalamin R binder is de novo synthesized in gastric cancer cells and that its plasma level increases in some patients. This binding protein may be a useful diagnostic and therapeutic parameter.

INTRODUCTION

Since an early report by Waxman and Gilbert (1, 2) that patients with hepatocellular carcinoma may have an extraordinarily high serum vitamin B12 (Cbl) binding capacity (UBBC), several studies have suggested the presence of CblBP in various malignancies with liver involvement (3–8). In gastric cancer, a single case with liver metastasis has been documented, in which levels of UBBC and TC I in the serum were elevated to more than 100 times the normal level (3). However, little is known about the causal relationship between tumor load and the high level of CblBP in plasma.

In the human body there are several CblBPs (9–12): (a) gastrin intrinsic factor that mediates B12 absorption from the small intestine; (b) plasma TC II, which facilitates cellular uptake of B12 by tissues; and (c) R binders, which are found in several body fluids and tissues. Plasma TC I and TC III have been considered immunologically equivalent to R binder.

For measurement of specific CblBPs, fractionation of binding proteins, ligand binding study (UBBC assay), and endogenous (saturated) B12 assays have been performed (13, 14). These are, however, cumbersome, and some problems are encountered in determining the actual cobalamin level (15). Therefore, with hybridoma techniques (16), we have developed an immunoassay that enabled direct measurement of both Cbl saturated and unsaturated binder with high sensitivity. Simultaneously, we have examined immunological properties of this binding protein and studied, in vitro and in vivo, whether gastric cancer cells produce CblBP.

MATERIALS AND METHODS

Tissue Binder Extraction. Stomach tissue was obtained from a 56-yr-old female with gastric cancer. Immediately after surgery, separate homogenates were prepared from the carcinoma itself and the mucosa adjacent to the tumor. The latter, histologically not involved in cancer, was used as a control in the gel filtration study, although it contained a small metastatic area. Homogenate was also prepared from the resected stomach of a 22-yr-old male with a perforated duodenal ulcer. This specimen contained neither metastatic area nor cancer cells. Extraction buffer consisted of 50 mM Tris-HCl (pH 7.5) with 0.25 M sucrose, 5 mM EDTA, and 5 mM ethyleneglycol bis(beta-aminoethyl ether)-N,N',N''-tetraacetate acid, 2 mM phenylmethylsulfonyl fluoride, 2 mM pepstatin, and 0.5% Triton X-100. Homogenates were gently stirred for 6 h at 4°C and centrifuged at 20,000 × g for 1 h. Supernatants were stored at −80°C. All specimens used were obtained with the patient’s prior consent.

Serum Deprivation Culture. We obtained Cbl-unsaturated binder (apo-binder) from serum- and Cbl-free medium of a gastric cancer cell line KATO-III, which was generously provided by Dr. M. Sekiguchi, Tokyo University, Institute of Medical Science (17). The cell line was established from poorly differentiated adenocarcinoma, and the presence of signet ring cells was noted. The cells were maintained in suspension in medium consisting of 45% DMEM, 45% RPMI-1640, 10% heat-inactivated fetal calf serum, 200 mM/liter of glutamine, streptomycin (100 µg/ml), and penicillin G (100 units/ml). Cells were split at a 1:10 ratio at weekly intervals. After subconfluent growth, cells were harvested, checked for viability, washed 3 times in Hank’s balanced salt solution, and then cultured again at a density of 1 × 106/ml in SSFM which consisted of DMEM containing 5 µg/ml of insulin (Sigma, St. Louis, MO), 5 µg/ml of transferrin (Sigma), 1% nonessential amino acids (Flow Laboratory), and antibiotics (18, 19). Spent medium was taken after 1 wk, concentrated about a hundredfold using an Amicon membrane (YM-10), and used for further study. There were no striking changes in the ratio of cell viability after 1 wk in SSFM. Conditioned SSFM containing Hela cells (CCL-2; American Type Culture Collection) and Intestine 407 (CCL-6) were used as controls.

Collection of Saliva and Plasma. Saliva was collected from healthy individuals and centrifuged at 10,000 × g for 20 min at 4°C, and the supernatant was stored. EDTA plasma was taken from patients with surgically confirmed gastric cancer (n = 72) and healthy individuals (n = 40). The state of metastasis was determined by examination before and during surgery. Plasma was also collected from patients with polycythemia vera (n = 2) and benign gastrointestinal disease (n = 30). The latter included cholecystitis (10), chronic and acute pancreatitis (7), gastrointestinal polyps (6), and gastric or duodenal ulcer (7).

Plasma was taken before and after surgery from 6 patients with gastric cancer and 3 with nonmalignant diseases (Cushing’s syndrome, aortocoronary by-pass operation, and pancreatic cyst). None of these patients had clinical evidence of bone marrow abnormalities except for two with...
polycythemia vera. All samples were stored at -80°C and not thawed until use.

**Assay of Vitamin B\textsubscript{12} and Cobalamin Binding Capacity.** Cobalamin unsaturated binders were measured by a modified albumin-coated charcoal method (14, 20). Saturated binders present in gel-fractionated materials were calculated from the endogenous Cbl determined by radioassay (Imnomphase; Cornling, Cornling, NY). The results were expressed as the concentration of binder-equivalent cobalamin (pg/ml).

To estimate the level of TC-II contamination in the materials, the UBBC assay was carried out at various pHs in PBS, and endogenous cobalamin was measured after adsorption of TC-II by silica (fumed; Sigma) (21). Recovery in both assays was evaluated.

**Gel Filtration Analysis and Purification of CblBP.** Conditioned media of various cell lines and stomach extracts were saturated with appropriate amounts of [\textsuperscript{57}Co]cobalamin (CT:2; Amersham) and applied to a Sephadex G-200 column (13, 22). Immunological cross-reactivity was examined by incubation of the radiolabeled materials with antisera before column chromatography, antigen-antibody reaction being detected by a shift of the appropriate binder peak toward the void fraction, thus indicating formation of a large complex (14). For further purification of binding protein, Cbl-Sepharose affinity chromatography was carried out (23).

**IEF.** Polyacrylamide rod gel electrophoresis, with a pH gradient of 2.0 to 5.0, was accomplished by modification of the method described by Laas et al. using Pharmalyte (Pharmacia, Uppasa, Sweden) (24). Before application to the gel, samples were labeled with [\textsuperscript{57}Co]Cbl; and fractionated on a Sephadex G-200 column. After optimal focusing was obtained, the gel was sectioned into 5-mm slices and assayed for radioactive B\textsubscript{12}. The pH gradient across each gel slice was determined by eluting with distilled water (1 ml) for 24 h and measuring the pH values of the eluents directly.

**Preparation of Polyclonal and Monoclonal Antibodies.** Serum of patients with pernicious anemia was used as anti-intrinsic factor antibody. RAR was raised against affinity-purified saliva R binder (25). MABs were prepared by immunizing BALB/c mice, separately, with affinity-purified saliva R and CblBP purified from conditioned SSF M KATOH-III (K-SSFM). Hybridization was carried out according to the modified method of Herzenberg et al. (26), using P3-NS1-1-Ag4-1 as a fusion partner and polyethylene glycol (M, 1500; BDH Chemicals, Poole, England). Hybridomas were cloned more than twice by limiting dilution. The screening assay was performed by EIA, as described below. These cloned hybridomas were then injected into pristane-primed BALB/c nude mice for ascites production, and the resulting antibodies were fractionated by anion exchange chromatography (Whatman; DE32).

**EIA.** For screening hybridomas, EIA was prepared as follows. Polystyrene microassay plates (Falcon No. 3912) were coated with 50 ml per well of RAR (20 µg/ml in PBS) and left overnight at 4°C. After being washed 3 times with T-PBS, the plates were blocked by using BSA-PBS for 1 h at room temperature and washed again. Fifty µl of partially purified saliva R or K-SSF M (50 µg of protein/well) were incubated for 2 h and washed with T-PBS. Fifty µl of culture supernatant of each hybridoma were then incubated for 2 h at room temperature and washed again in T-PBS. Bound antibody was determined using 50 µl of affinity-purified goat F(ab')\textsubscript{2} antihorse IgG (TAGO 4550) at a 1:1500 dilution in BSA-PBS (27, 28). Color was developed with 0.04% o-phenylenediamine in 0.1 m citrate buffer (pH 5.0) containing 0.006% H\textsubscript{2}O\textsubscript{2}. Hybridomas from mice immunized with CblBP from K-SSF M were screened by dual antigens in each cloning procedure, first against K-SSF and then against saliva R, to exclude clones that produced antibody reactive to a shared epitope.

**Immunoperoxidase.** Saliva, K-SSF, normal plasma, gastric cancer extract, and extracts of noncancerous mucosa both with and without metaplasia were radioiodinated by the lactoperoxidase method (29). Radiolabeled materials were then incubated with RAR or MABs and immunoprecipitated by adding Protein A-Sepharose (Pharmacia) and leaving overnight at 4°C according to a previously described procedure (30). Final precipitates were analyzed by both SDS-PAGE and autoradiography using Kodak XAR film exposed for 1 wk at -80°C. [\textsuperscript{57}Co]-B\textsubscript{12} affinity-labeled saliva R and K-SSF were simultaneously incubated with various antibodies and immunoprecipitated similarly. After several washings, the radioactivity in the final precipitates was counted with a well-type gamma counter.

**Chemical and Enzymatic Treatment of Antigens.** KATOH-III in PBS was disrupted by sonication and then centrifuged at 100,000 x g for 30 min with the resulting supernatant being used for antigen preparation. Digestion by Pronase (EC 3.4.24.4; Pronase E from Strep tomyces; Kaken Kagaku, Tokyo, Japan) was carried out in 0.05 M Veronal buffer (pH 7.7) at 37°C for 46 to 48 h at an enzyme-substrate ratio of 1:40 (31). For destruction of carbohydrate structure, antigen preparations were treated with neuraminidase (EC 3.2.1.18; \textit{Vibrio cholerae} neuraminidase; Behringwerke AG, Marburg, West Germany) and then with periodate according to the method of Coligan and Todd (32). Neuraminidase (1 unit/ml) was added in a ratio of 1 millilitre to 60 µg of each antigen. Periodate oxidation was performed using sodium metaperiodate (Nakarai Chemicals, Kyoto, Japan), in a final concentration of 5.33 mmol, at room temperature and in the dark for 40 h. The reaction mixture was then intensively dialyzed against PBS. Reactivities of treated antigens to MABs 55-D, WK-1, and H-12 were investigated using solid-phase RIA in which assay plates were precoated with 100 µl of each MAB (30 µg/ml) and blocked by BSA-PBS. After incubation with treated cell lysate of KATOH-III, bound antigen was detected by radioiodinated RAR (approximately 3 x 10\textsuperscript{5} cpm). Using the same precoated assay plates, competitive inhibition was also studied. Briefly, to a constant amount of radioiodinated cell lysate of KATOH-III, we added increasing amounts of cold inhibitors, which were treated lysates of the same cell, untreated lysates of granulocytes (5.0 mg of protein/ml), and purified plasma TC I and TC III. After aspiration of the radiolabeled materials, the plates were washed extensively, and the radioactivity of each separated well was determined by a gamma counter.

**Assay for Patient Plasma.** For the CblBP assay in the patient plasma, a sandwich-type EIA was developed. Briefly, assay plates were coated with 50 µl of MAB 55-D or H-12 (20 µg/ml) and blocked with BSA-PBS. After washing with T-PBS, 50 µl of plasma, diluted 1:2 with PBS, were incubated. Fifty µl of gastric cancer extracts in a dilution series from x10 to x10\textsuperscript{2} were used as an assay standard. Bound antigen was detected by adding 50 µl of RAR (Fab') which was labeled with horse radish peroxidase by the maleimide method (33). Immunoreactive binder contained in the extract at 5 x 10\textsuperscript{2} dilution was tentatively assumed to have a concentration of 1 unit/ml; this value was approximately equivalent to 0.13 µg of cobalamin/ml, determined by Sephadex G-200 chromatography and the binder assay described earlier. Unknown samples were quantified from the calibration curves. For statistical analysis, the Student t test was used.

**Immunostaining.** Sections of formalin-fixed and paraffin-mounted cancer tissue, normal stomach tissue, and KATOH-III smears were stained with MAB 55-D and H-12 as follows. Sections with paraffin removed and cell smears were treated with 0.3% hydrogen peroxide in absolute methanol for 20 min in order to block endogenous peroxidase activity. After blocking the nonspecific binding of immunoglobulin by incubation in 30% normal horse serum for 1 h, the sections and cell smears were further incubated with MAB 55-D and H-12 at a 1:2000 dilution overnight at 4°C. Samples were washed with PBS and incubated for 2 h at 4°C, with biotinylated anti-mouse immunoglobulins (Vector, Burlingame, CA) at a 1:500 dilution in BSA-PBS containing 2% normal human serum. Following washing with PBS, the sections and smears were incubated with horseradish-avidin D (Vector) for 1 h at 4°C. The histochemical determination of peroxidase was carried out according to the modified method of Graham and Karnowsky (34). Nonimmunized mouse serum was used as the negative control.

**RESULTS**

**Gel Filtration Analysis and Binding Study.** In extracts from either cancerous or control tissues, UBBC was less than 10% of the total binding capacity. A large peak representing a M, 125,000 binder was observed in both cancer extract and K-SSF M (Fig. 1, D and G). On the other hand, binders caused a
$M$, 80,000 to 90,000 broad-based peak with a small $M$, 125,000 shoulder in the control extract (Fig. 1A). Free Cbl was eluted at the end of the column. UBBC was markedly elevated in K-SSFM (Fig. 1G), but was almost at background level (10 to 20 pg/ml) in the case of Hela cells and Intestine 407 (data not shown). UBBC in K-SSFM became undetectable when cycloheximide (5 μg/ml) was added in the culture medium.}

**Fig. 1.** Elution profiles of [$^{57}$Co]B$_{12}$-labeled with and without antisera. Labeled samples (1 ml) were applied to a column (1.4 x 28 cm) of Sephadex G-200, allowed to equilibrate, and then eluted with 0.05 m potassium phosphate (pH 7.4) containing 0.5 m NaCl. Fractions of 2.5 ml were collected, and UBBC and endogenous cobalamin were assayed. Apparent molecular weights were estimated from a calibration kit. A, control extract; B, control extract with anti-R antibody; C, control extract with anti-IF antibody; D, cancer extract; E, cancer extract with anti-R antibody; F, cancer extract with anti-IF antibody; G, conditioned SSFM with anti-IF antibody; H, K-SSFM with anti-R antibody; I, K-SSFM with anti-IF antibody. O, UBBC; •, endogenous cobalamin including [$^{57}$Co]B$_{12}$ added; A, UBBC when cytoplasmatic; B, control extract with anti-R antibody; C, control extract with anti-IF antibody; D, cancer extract; E, cancer extract with anti-R antibody; F, cancer extract with anti-IF antibody; G, conditioned SSFM with anti-IF antibody; H, K-SSFM with anti-R antibody; I, K-SSFM with anti-IF antibody. O, UBBC; •, endogenous cobalamin including [$^{57}$Co]B$_{12}$ added; A, UBBC when cytoplasmatic.

**Fig. 2.** Isoelectric focusing of [$^{57}$Co]B$_{12}$-labeled, gel-fractionated samples. Electrophoresis was started with an initial voltage of 200 V for 1 h, then 300 V for 5 h, and finally 400 V for 1 h, A, cancer extract; B, K-SSFM; C, saliva R; D, extract of control mucosa with intestinal metaplasia. O, pH of the eluant of sliced gel; •, pH of the eluant of sliced gel.

IEF. Isoelectric points (pI) of the binder in cancer extract were distributed between 2.8 and 3.8. The pIs determined were: 3.2 in K-SSFM; 3.6 in the control extracts (from noncancerous mucosa with metaplasia); 5.0 in the extract from a young patient without metaplasia (data not shown); and, for reference, 3.8 to 4.3 in saliva R (Fig. 2).

Immunoprecipitation. Despite different origins, an essentially identical $M$, 70,000 group was immunoprecipitated in all samples, except for the extract obtained from one young patient (Fig. 3).

Reactivity with Monoclonal Antibodies. Of 50 clones from the 700 positive wells resulting from 5 consecutive hybridizations in mice immunized with K-SSFM, 12 clones were retained after the cloning procedure. Among these clones, WK-1 (IgG3) and H-12 (IgG3) were used for further study. Clones 55-D (IgG1) and 42-C (IgG3) were derived from mice immunized with saliva R. All these MAbs immunoprecipitated $M$, 70,000 antigen in cell lysate of KATOH-III (Fig. 4A); however, MAbs WK-1 and H-12 did not react with saliva R (Fig. 4B). The findings were further confirmed by an additional radioimmunoprecipitation assay using [57Co]B$_{12}$ (Table 1). Binding of MAbs to this $M$, 70,000 antigen was reduced by Pronase treatment in 55-D and by periodate and neuraminidase treatment in 55-D. In WK-1 and H-12, especially the latter, these bindings were competitively inhibited by untreated antigen; however, inhibition was abolished by Pronase treatment in 55-D. In WK-1 and H-12, especially the latter, binding was inhibited either by neuraminidase treatment alone or with periodate (Fig. 5). In summary, the epitope of 55-D appeared to be a peptide, while those of H-12 and WK-1 appeared to be carbohydrate moieties (containing terminal sialic acid) of cancer-derived binders.
cytosol by 55-D, but not by H-12 (data not shown).

**Plasma Assay.** Fig. 8A shows the calibration curve of the EIA, of which sensitivity was estimated to be $10^{-15} \text{M}$, about 100 times higher than that of gel filtration combined with endogenous cobalamin assay. Since the inhibition study indicated cross-reactivity of 55-D to TC I and TC III (Fig. 8B), we used H-12 in the plasma assay. Cancer patients, even those without liver metastasis, showed significant levels of immunoreactive binder (5.5 ± 0.96 units/ml; mean ± SE) compared to healthy controls (2.5 ± 0.07 units/ml) (Fig. 9). The differences of WBC count between these two groups were not significant (data not shown). In 4 cases without metastasis, originally elevated levels of R binder were decreased subsequent to tumor excision, whereas, in 2 patients who underwent gastrojejunostomy necessitated by liver and peritoneal metastasis, binder levels remained high (Fig. 10). Levels of R binder were not affected by surgery in 3 cases of nonmalignant disease.

**DISCUSSION**

Since granulocytes at some differentiation stage produce CblBP of the R binder class (TC I and TC III) (35, 36), increased concentrations of transcobalamin have been noted in some myeloproliferative disorders (36–41). In addition to the total-body granulocyte pool and granulocyte turnover (42), several
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Fig. 7. Immunoreactive R binder stained by 55-D (a and c) and H-12 (b); papillary (a) and signet cell (b) carcinoma of the stomach; (c) intestinal metaplasia of the stomach.

Fig. 8. A, calibration curves of EIA for quantification of immunoreactive R binder in plasma. Ordinate, absorbance; abscissa, dilutions of cancer extract. O, 55-D; O, H-12. B, cold inhibition study using radioiodinated lysate of KATO III and granulocyte lysate. □, 55-D; □, H-12. A representative experiment is shown. Points, mean of triplicate determinations. The result when using plasma TC I and TC III purified with Sephadex G 200 was essentially the same.

Fig. 9. Immunoreactive R binder in plasma detected by EIA using H-12 and RAR. H (+), cancer patients with liver metastasis; H (−), cancer patients without liver metastasis; **, P < 0.01; *, P < 0.05. The solid lines and dashed line are mean values and the mean ± 2 SD of control, respectively.

Fig. 10. Immunoreactive R binder detected by H-12 in plasma before and after surgery. A, B, C, and D, gastric cancer patients who had cancer excised; E and F, gastric cancer patients who had by-pass surgery because of advanced metastasis and pyloric stenosis; G, H, and I, control patients who underwent surgery for pancreatic cyst, Cushings' syndrome, and myocardial infarction, respectively.

reports have indicated other factors which affect the concentrations of plasma R binder (6, 43–45), particularly in the neutropenic state (43) or in some malignancies without bone marrow abnormalities (3–8). Except in adolescent-type hepatoma (4), direct evidence for the production of CblBP by cancer cells has been insufficient to date. Using a serum-free, in vitro system, we observed an increase of CblBP in the culture supernatant of a gastric cancer cell line. This increase was inhibited by the presence of cycloheximide. In extracts from cancerous tissue, the level of a M, 125,000 binding protein was elevated. Histochmical studies showed this binding protein was localized in cancer cells. The level of immunoreactive CblBP in plasma was significantly elevated in cancer patients, even if neither liver
nor bone marrow metastasis was present. A subsequent decrease in concentration after tumor excision was also observed. Together, all these lines of evidence lead us to conclude that the binder was produced by the de novo synthesis of gastric cancer cells and was not the result of the release of preexisting tissue stores of CbBP secondary to tumor invasion.

Although some heterogeneity in charge was observed, the biochemical properties and immunological cross-reactivity of these CbBPs were very similar to those of R binders. The discrepancy in molecular weight between SDS-PAGE and gel filtration has also been documented (25, 46). Conventionally, characterization of R binders from various sources has been performed by anion exchange chromatography (14) and isoelectric focusing (47); however, these procedures are impractical for routine examination. Since microheterogeneity of R binders from various sources has been ascribed to differences in carbohydrate moieties (25, 48, 49), we speculated that such minute differences in carbohydrate residue might lead to the definition of a new group of epitopes. Using pooled saliva R as the standard antigen for normal “glandular”-type R binder (47) and eliminating clones reactive to shared epitopes, we were able to obtain hybridomas producing antibodies reactive to determinants of binders from cancer cells. Our findings suggested a higher sialic acid content in CbBP of K-SSFPM than in saliva R.

Our preliminary study showed that no significant difference was observed in plasma B12 and UBBC levels between the patients and control groups by radioassay, apparently because of the varying amounts of free cobalamin and TC II present. Neither the UBBC nor B12 level was, therefore, informative for determining total (holo- and apo-) R binder levels. Immunoassay using 55-D, which measures total immunoreactive R binder, disclosed markedly increased levels of this binding protein in two cases of polycythemia vera (395 units/ml and 330 units/ml) and a significant increase in cancer patients with liver metastasis. However, the difference of mean values between gastric cancer patients without liver metastasis and patients with benign disease was not significant. Using the H-12 assay, which measures a carbohydrate epitope that is probably more associated with cancer cells, this difference has become significant, and the values obtained in polycythemia vera were both less than 20 units/ml. Since we observed a considerable increase of immunoreactive R binder in one patient with cholecystitis and obstructive jaundice, we think that delayed hepatic clearance of R binder (50, 51) might have contributed to the increase in this case.

It is of considerable interest that positive staining was confined to cancer cells and cells involved in intestinal metaplasia. Using polyclonal antibodies, we recently demonstrated that immunoreactive R binder is not present in normal stomach mucosa but is found in goblet cells of the colon and surface epithelial cells of the small intestine (52). In the present study, R binder with a molecular weight of 70,000 was immunoprecipitated in the extracts from carcinoma and mucosa with intestinal metaplasia, but not in the extract without metaplasia. Therefore, binder production by the gastric mucosa suggests some histogenetic background common to intestinal metaplasia and some types of gastric cancer (53).

Although a high level of R binder in plasma is not specific to gastric cancer, its decrease after therapeutic intervention may be useful in clinically monitoring the course of the disease.

ACKNOWLEDGMENTS

The authors wish to acknowledge the help of the following people: Y. Okada, Department of Physiology, Kyoto University; K. Kuribayashi, Institute of Immunology, Kyoto University; and S. Miyata, Takeda Hospital, Kyoto; and would also like to thank S. Nishikawa of Kumamoto University, E. Tatsumi of Kobe University, R. Kannagi of Kyoto University, and N. Tanaka of Jikei Medical College.

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