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An Alternative Form of IL-18 in Human Blood Plasma: Complex Formation with IgM Defined by Monoclonal Antibodies

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Monoclonal Abs 21 and 132 were raised against human functionally inactive rIL-18, and plasma IL-18 levels were determined by the sandwich ELISA established with these mAbs. Plasma IL-18, designated type 2, was detected by this ELISA, and the levels found were not consistent with those obtained with the commercially available kit for determination of functionally active IL-18 (type 1). Type 1 was detected in all volunteers, whereas type 2 was detected in ~30% of healthy subjects, and the levels of type 2 in their blood plasma were high (25–100 ng/ml) compared with those of type 1 (0.05–0.3 ng/ml). We purified IL-18 type 2 from blood plasma of volunteers with high IL-18 type 2 concentrations, and its Mr was determined to be 800 kDa by SDS-PAGE and molecular sieve HPLC. The purified 800-kDa protein, either caspase-1-treated or untreated, expressed no or marginal IL-18 function in terms of poteniation of NK-mediated cytolysis and IFN-γ induction, and it barely bound IL-18R-positive cells. N-terminal amino acid analysis indicated that the purified protein was IgM containing a minimal amount of IL-18 proform and its fragment. Again, the purified IgM from IL-18 type 2-positive volunteers exhibited cross-reaction with mAb 21 against IL-18. This band was not detected with 125-2H, an mAb against functionally active IL-18. Hence, human IgM carries functionally inactive IL-18 forming a disulfide-bridged complex, and this IL-18 moiety is from 10- to 100-fold higher than the conventional type 1 IL-18 in blood circulation in ~30% normal subjects. The Journal of Immunology, 2001, 166: 6671–6679.

interleukin-18, formerly called IFN-γ-inducing factor (1), is a cytokine structurally similar to IL-1β (2). IL-18, as well as IL-12, is produced during the acute immune response by macrophages (Mφ) and immature dendritic cells (DC) as an initial cytokine and acts on target receptor-positive cells (3). Like IL-1β, IL-18 is a cytoplasmic protein synthesized as a biologically inactive 24-kDa precursor molecule lacking a signal peptide that requires cleavage into an active, mature 18-kDa molecule by the caspase-1; Refs. 4 and 5). However, the mechanism of secretion from IL-18 of Mφ has not been clarified. IL-18 serves as a soluble ligand for the IL-18R complex on NK and T lymphocytes to enhance a lymphocyte-mediated immune response (6).

IL-18 solely up-regulates Fas ligand and perforin in NK and T cells to facilitate target cell killing (7). The most important role of this cytokine is as a costimulant for IL-12, which induces the Th1 response, primarily by its ability to induce IFN-γ production (1, 6, 7). Taken together with IFN-γ, they act on T cells and sustain a Th1-dominant immune environment (1, 7). In the allergic mouse model, Ab production including IgE was suppressed by simultaneous administration of IL-12 and IL-18 (8). These functions of IL-18 were evident even in the absence of Ag stimulation and cell-to-cell contact. Usually, intrinsic soluble mediators or extrinsic microbes provoke innate immune activation that provides an essential basis for lymphocyte activation by APCs, i.e., Mφ and DC (3). Although most of these results were obtained with the mouse system, it is accepted that human IL-18 is a functional homologue of mouse IL-18 (9).

Recently, a number of surprising findings on IL-18 have been reported (5, 10). Murine/human Mφ liberate a precursor form of IL-18 (proIL-18). Also, unlike IL-1β, IL-18 is constitutively expressed as the level of mRNA (1, 5). In addition, although the precursor form of IL-1β (proIL-1β) and proIL-18 are substrates for caspase-1 (10, 11), the majority of proIL-18 remains unprocessed in cells even under conditions where sufficient caspase-1 is provided (5). There are a number of possibilities to explain the last result. First, proIL-18 may form an intracellular complex with other proteins similar to extracellular IL-1β (12, 13), IL-6 (14), and IL-2 (15) to circumvent protease cleavage. Second, proIL-18 may be essentially less sensitive to caspase-1 than proIL-1β (10, 16). Last, proIL-18 may consist of a protease-sensitive form and a protease-insensitive form, which can be generated by various post-translational modifications (17). Indeed, intracellular processing of proIL-18 has been shown to be different from that of proIL-1β (5, 18), even though these two cytokines share the same activating enzyme, similar structural properties, and similar intracellular localizations (2, 4).
Our initial data (17) suggested that large amounts of IL-18 were present in the blood plasma of normal subjects by using the polyclonal Ab (pAb) against the inactive form of rIL-18. This was similar to a recent report (5) suggesting that human Mφ secrete soluble 24-kDa pro-IL-18. To clarify this issue, we produced mAbs against our inactive rIL-18 (19) and measured the levels of IL-18 in human plasma by a novel sandwich ELISA. Comparison of our data to those obtained with the conventional ELISA for measuring the active form of IL-18 (20) brought about large discrepancies in the plasma levels of IL-18. Strikingly, a tremendous amount of unidentifiable IL-18 was found to exist in some of the normal subjects (17). The present study demonstrates the presence of a unique IL-18 isotype in normal human plasma and that this isotype can be defined by our mAbs and form a complex with IgM. Our present results may offer a hint to explain the message level-to-protein level inconsistency in the studies of IL-18. The frequency and structural and functional properties of this form of IL-18 will be analyzed in this report.

Materials and Methods
Blood samples, cells, and reagents
Fresh blood plasma was prepared from the heparin- or citrate phosphate dextran (CPD)-supplemented blood (20 U/ml) of volunteers (age 25–65 years) by centrifugation (2000 x g, 10 min). All volunteers were found to be healthy in our clinic. The active rIL-18 preparation was a kindly gift of H. Okamura (Hyogo Medical College, Nishinomiya, Japan) and was used for the reported functional studies (9). The inactive rIL-18 preparation was produced by the method of Kohler and Milstein (23). The cDNA of this form was cloned into an expression vector PHP-L3, and a rIL-18 protein with an N-terminal 6-histidine tag was expressed in the GTF24 strain of Escherichia coli. (19). The protein was purified by a nickel column as described previously (19). Female BALB/c mice were immunized with the purified Ag (5 μg) plus CFA every 7 days for 28 days. Finally, 20 μg of Ag was administered i.p. as a booster. Three days later, the spleen was extracted, and the cells were fused with the mouse myeloma cell line NS-1 (24). The supernatants of hybridomas were screened by ELISA and immunoblotting with the recombinant Ag as described previously (25). Six clones producing mAbs that reacted with the recombinant protein were established by limiting dilution. Three mAbs (21, 132, and 355) that reacted well with inactive rIL-18 were purified from mouse ascites by ammonium sulfate precipitation followed by protein G-Sepharose (Amersham Pharmacia Biotech.), and HRP-conjugated goat anti-mouse IgG and HRP-labeled anti-rabbit IgG were from Bio-Rad (Hercules, CA). A pAb against our inactive rIL-18 (19) and measured the levels of IL-18 for the reported functional studies (9). The inactive rIL-18 preparation was a kind gift of Dr. H. Okamura. Plasma samples were collected from patients with acne vulgaris (20) by centrifugation (2200 x g, 10 min). Caspase-1 was purchased from Calbiochem (lot no. B32552; La Jolla, CA). A peptide inhibitor specific for caspase-1 (Ac-Tyr-Val-Ala-Asp-chloromethylketone) was purchased from Takara (Shiga, Japan). Human PBMC were prepared from CPD-supplemented human blood as reported previously (19, 21). A Hodgkin’s lymphoma cell line, L428, was a gift from Dr. K. Orita (Fujisaki Cell Center, Okayama, Japan; Ref. 22). A human macrophage-like cell line, THP-1 and other human cell lines were provided by Human Science Research Resource Bank (Osaka, Japan).

Production of Abs against human IL-18 and IL-18R
A pAb directed against the inactive rIL-18 was prepared as described previously (19). The Ag used for the production of pAb was the 18-kDa form of human rIL-18 with a 6-histidine tag (19). mAbs against this same IL-18 Ag were produced by the method of Köhler and Milstein (23). The cDNA of this form was cloned into an expression vector PHP-L3, and a rIL-18 protein with an N-terminal 6-histidine tag was expressed in the GTF24 strain of Escherichia coli. (19). The protein was purified by a nickel column as described previously (19). Female BALB/c mice were immunized with the purified Ag (5 μg) plus CFA every 7 days for 28 days. Finally, 20 μg of Ag was administered i.p. as a booster. Three days later, the spleen was extracted, and the cells were fused with the mouse myeloma cell line NS-1 (24). The supernatants of hybridomas were screened by ELISA and immunoblotting with the recombinant Ag as described previously (25). Six clones producing mAbs that reacted with the recombinant protein were established by limiting dilution. Three mAbs (21, 132, and 355) that reacted well with inactive rIL-18 were purified from mouse ascites by ammonium sulfate precipitation followed by protein G-Sepharose purification according to the manufacturer’s protocol (Amersham Pharmacia Biotech, Piscataway, NJ).

Sandwich ELISA for determination of plasma IL-18
Plasma IL-18 concentrations were determined by the two systems of sandwich ELISA for determination of active and inactive rIL-18 (19). The former was measurable by the commercially available kit. The latter was first introduced in this study. Briefly, all mAbs were purified from mouse ascites by ammonium sulfate precipitation followed by protein G-Sepharose (Amersham Pharmacia Biotech.), and combinatorial studies were performed for development of the sandwich ELISA (25). HRP-conjugated mAbs were prepared by the NaIO4 (24) and used as detection Abs. Next, 100 μl (1–2 μg) of capture Ab or control mouse nonimmune IgG (1–2 μg) was added to each well of 96-well ELISA plates (Dynatech Laboratories, Chantilly, VA), and allowed to stand for 4–20 h at 4°C. The wells were washed with an ELISA washer (Bio-Rad), then 50 μl of each plasma sample and 50 μl of PBS containing 10% BSA and 10 μg of detection Ab were added to the wells. Two hours later, the wells were aspirated and washed three times with saline containing 0.005% Tween 20, and then 100 μl of color reagent (0.1% tetramethyl benzidine in 100mM phosphate-citric buffer containing 0.006% H2O2 (pH 5.0)) was added. In ~30 min, the reaction was stopped with 100 μl of 1 M sulfuric acid. The absorbance at 450 nm (A450) was measured with a microplate photometer (MTP-120; Corona Electric, Tokyo, Japan).

For reference, the sandwich ELISA kit (MBL-Immunotech) was used for determination of active IL-18 according to the instruction booklet.

Purification of the plasma IL-18 defined by our mAbs
The pAb (20 ng) against inactive IL-18 was conjugated to Affigel 10 (10 ml) (Bio-Rad, Hercules, CA) as described previously (25). The sheets were sequentially incubated with mAb (10 μg/ml) plus CFA every 7 days for 28 days. Finally, 20 μg of detection Ab were used, the detection limit of plasma IL-18 being 0.5 ng.

For immunoblotting, purified materials of IL-18 were mixed with lysis buffer (1% Nonidet P-40, 10 mM EDTA, 25 mM iodoacetamide, 2 mM PMSF, Dulbecco’s PBS) for 20 min at room temperature to adjust the concentration to 10 μg/ml. Samples were boiled for 5 min in the presence of 3% of SDS and 0.3 M of 2-ME for reduction. Aliquots (50 μl) of the samples were subjected to SDS-PAGE (12.5% gel) under either nonreducing or reducing conditions. After electrophoresis, the resolved proteins were transferred onto nitrocellulose sheets. The sheets then were blocked with 10% skim milk for 1 h at 37°C and allowed to stand overnight at 4°C. The sheets were sequentially incubated with mAb (10 μg/ml) and HRP-conjugated goat anti-mouse IgG (1 μg/10 μl), followed by staining with an ECL kit (Amersham Pharmacia Biotech) as described previously (19).

Flow cytometric analysis was performed as described previously (20). Briefly, 25 ng of IL-18 type 2 or control IgM was incubated with 105 cells for 40 min at 4°C. After three washes with PBS, IL-18 binding was detected with anti-IgM Ab (10 ng) and FITC-labeled second Ab. Samples were analyzed by FACScalibur (Becton Dickinson, Mountain View, CA) within 2 h.

Determination of amino-terminal amino acid sequence of IL-18
N-terminal sequences of purified IL-18 species were determined by using a peptide sequencer (Shimazu 2700; Shimazu, Tokyo, Japan). Blot sheets were stained with commassie blue, and the stained bands were cut for...
analysis of N-terminal sequences. The sequencing of the region of IgM in nonreduced samples and the bands of 24 kDa and 17 kDa in reduced samples was conducted three times. The sequence other than those of the \( \mu \)- and \( \kappa \)-chains are shown as the third sequence in the nonreduced IL-18 type 2 samples.

**Caspase-1 treatment of IL-18 type 2**

A stable transfectant expressing human proIL-18 with a 6-histidine tag was established with the rabbit kidney cell line RK13. Cells were maintained in DMEM containing 10% FCS. ProIL-18 with a 6-histidine tag was purified from the cells by a nickel column (our unpublished data). One microgram of proIL-18 or IL-18 type 2 was incubated with 25 U of caspase-1 for 3 h at 25°C in 50 \( \mu \)l of the buffer (20 mM HEPES, 8% glycerol, and 1 mM DTT) recommended in the manufacturer’s booklet. The caspase-1 inhibitor was used to test protease specificity. Over 95% of the caspase-1 activity was blocked with 900 \( \mu \)M of this peptide inhibitor. The IL-18 samples were analyzed on SDS-PAGE and immunoblotting.

**Assay for IFN-\( \gamma \)-inducing activity and NK-mediated cytolysis**

Human PBMC were collected with methylcellulose sedimentation followed by centrifugation on a Ficoll-Hypaque cushion (19). The cells at the interphase were collected and washed twice with RPMI 1640/10% FCS (21). Aliquots (3 \( \times \) 10^5 PBMC) of these preparations were incubated with IL-12 (10 ng/ml) plus active rIL-18 (40 ng/ml) or IL-18 type 2 (25–100 ng/ml) in 96-well plates, and 48 h later, the supernatants were collected.

**Results**

**Properties of mAbs against human inactive rIL-18**

Rabbits were immunized with the inactive form of rIL-18 Ag that was produced in E. coli (GIL24) and formed monomers and disulfide-bonded multimers (19). The resultant pAb recognized both the monomer and multimers of the inactive IL-18 as well as the active form of rIL-18 (Fig. 1A). Preliminary experiments suggested that this pAb but not 125-2H (an mAb for detection of active IL-18) recognizes a plasma protein by immunoprecipitation (data not shown). Thus, we decided to produce mAbs specifically recognizing the plasma protein antigenically related to IL-18. mAbs against inactive rIL-18 basically recognized both monomer and multimers (Fig. 1B). Of note, mAb 21 recognized reduced rIL-18 preparations. In contrast, the mAb produced against the active form of rIL-18 (MBL-Immunotech) allowed to detect only the monomeric but not the multimeric forms of both preparations and much less efficiently recognized the inactive rIL-18 monomer than the active rIL-18 monomer (Fig. 1B). Thus, the mAbs we

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**FIGURE 1.** Recognition of active and inactive rIL-18 by our established mAbs. A, Immunoblotting profiles of rIL-18 proteins with pAb. One hundred nanograms of the active (left lane) and inactive rIL-18 (right lane) were subjected to SDS-PAGE followed by immunoblotting. No nonspecific band was observed by probing nonimmune rabbit serum (right). The blot was probed with polyclonal antisera against inactive rIL-18 (left). HRP-labeled secondary Ab and ECL reagents were used for color development. B, Immunoblotting profiles of rIL-18 proteins with mAbs. The active (left lane) and inactive (right lane) rIL-18 preparations were compared on SDS-PAGE/immunoblotting as in A. A relatively slower mobility was observed in the inactive rIL-18 sample in the right lanes, probably due to the histidine tag. The probing mAbs are shown in the figure. C, Detection of active and inactive rIL-18 by ELISA with mAbs recognizing different epitopes. Wells in the 96-well plate were coated with capture mAbs and the active and inactive rIL-18 samples were added as indicated (x-axis). Each detection mAb (10 ng) was HRP-labeled and added to the wells together with 10% BSA, and color was developed with tetramethyl benzidine. The reactivity of each mAb with the active and inactive preparations was shown by A_450 (y-axis). Color development was for 30 min. D, Dose-response curve for measurement of inactive rIL-18. The indicated amounts of rIL-18 was measured by the ELISA we developed. The assay was repeated three times in triplicate and error bars were calculated. Color development was for 15 min.
produced may recognize distinct forms of human IL-18 not identified with the conventional mAbs produced against active rIL-18. Based on these results, we tried to establish an ELISA system for determination of the distinct forms of IL-18 in human plasma.

Establishment of ELISA with mAbs against the inactive rIL-18

We raised three mAbs recognizing the inactive rIL-18. The reactivity of these mAbs was assessed with both active and inactive rIL-18 preparations by SDS-PAGE/immunoblotting (Fig. 1B) and ELISA (Fig. 1C). Like the pAb, two mAbs (21 and 355) predominantly recognized the multimers of inactive rIL-18 in these two assays. Other mAbs recognized the two rIL-18 preparations in a similar fashion (data not shown). In contrast, the mAbs against active rIL-18, 125-2H and 25-2G, recognized active rIL-18 yet were unable to detect the inactive rIL-18 (Fig. 1C). These results suggest that the mAbs we produced recognized Ag epitopes that were distinct from those of the commercially available mAbs.

The sandwich ELISA system for inactive rIL-18 was established in our laboratory as shown in Fig. 1, C and D. The most sensitive was the use of mAb 132 as the capture Ab and mAb 21 as the detection Ab. This combination of mAbs detected ~0.5–200 ng/ml rIL-18 in a dose-responsive manner (Fig. 1D). Only the inactive form of rIL-18 was detected with our established ELISA, in contrast to the conventional ELISA for IL-18, which was specific for active IL-18 (Fig. 1, C and D).

Detection of IL-18 type 2 in human plasma by sandwich ELISA

Next, we applied the sandwich ELISA for quantitation of the plasma IL-18 with these mAbs (Fig. 2A and B). Our ELISA as well as commercial ELISA for active IL-18 detected an Ag in human plasma. We tentatively designated the plasma isotypes defined by the mAbs against inactive rIL-18 as IL-18 type 2 and the conventional IL-18 defined by the mAbs against active rIL-18 as type 1 and proceeded to characterize novel unusual IL-18 moieties by the produced mAbs.

With this ELISA system for IL-18 type 1 and type 2, we measured the levels of IL-18 in 12 plasma samples from normal subjects (Fig. 2A and B). Five of the 12 normal donors had levels of type 2 that were 25–100 ng/ml, whereas all donors had 20–220 pg/ml type 1 with reference to the ~18-kDa control rIL-18. Thus, some healthy subjects possessed from 10- to 100-fold more type 2 than the conventional type 1 IL-18 in their blood plasma. There was no correlation between levels of IL-18 type 1 and type 2 in these subjects (Fig. 2C).

To rule out the possibility that the IL-18 type 2 was an artifact produced during the preparation of blood plasma, we tested the effect of anti-coagulants and various storage periods on type 2 levels with two independent plasma samples. Concentrations of IL-18 type 2 in serum were similar to those in plasma (data not shown). In all cases, we were unable to detect any significant differences in type 2 levels by our ELISA system (data not shown).

Next, we tested cross-recognition of IL-18 type 1 and type 2 by the two ELISA systems. The commercial ELISA system detected only active and type 1 IL-18, whereas our ELISA system detected only inactive and type 2 IL-18, thereby discriminating between the two isoforms of IL-18 (Fig. 3). In conclusion, an alternative type of human IL-18 was defined by our mAbs. About 30% of healthy subjects possessed this type of IL-18 in their blood plasma, which is antigenically and structurally distinct from the conventional or active IL-18.

Purification of IL-18 type 2, forming a complex with IgM

Next, we attempted to purify the alternative IL-18 from human plasma containing high levels of type 2. During preceding molecular sieve HPLC analysis, we found that IL-18 type 2 reproducibly eluted at an ~800-kDa IgM region and other regions based on ELISA criteria (Fig. 4A). IL-18 type 2 was purified by using DEAE-Sephacel, anti-rIL-18 pAb-coupled Affi-Gel, and MonoQ columns from CPD-supplemented plasma of two individual donors (Fig. 4, B–D). Finally, the 800-kDa protein was eluted and detected with mAbs 21, 355, and 132 by immunoblotting (Fig. 5). With SDS-PAGE, the eluted protein aligned with human IgM under reducing and nonreducing conditions (Fig. 5D). Under reducing conditions, the 800-kDa moiety was reduced into 70-kDa and 27-kDa proteins by Coomassie blue-staining, suggesting heavy and light chains of IgM, both of which were not recognized by the mAbs 125-2H or 25-2G (Fig. 5). In contrast, by probing with mAb 21, the 800-kDa moiety was reduced into a faint 24-kDa band. This was recognized by mAb 21 and aligned with rIL-18 proform (not shown). Again, the 24-kDa protein was undetectable with
The ability of IL-18 type 2 to enhance IL-12-mediated IFN-\(\gamma\) induction was measured (Fig. 7A). Unlike active rIL-18, type 1 was reported to be a substrate for caspase-1, and in fact, M\(\beta\)B IL-18 type 2 (data not shown). IL-18 has been shown to enhance killing by IL-18 type 2. No enhancement of killing was observed with T cells. Next, we tested whether NK-mediated cytolysis was enhanced by IL-18 type 2. No enhancement of killing was observed.

Functional properties of IL-18 type 2

The ability of IL-18 type 2 to enhance IL-12-mediated IFN-\(\gamma\)-induction was measured (Fig. 7A). Unlike active rIL-18, type 2 did not enhance IFN-\(\gamma\)-production by lymphocytes containing NK and T cells. Next, we tested whether NK-mediated cytolysis was enhanced by IL-18 type 2. No enhancement of killing was observed with the addition of IL-18 sequence in the IgM region and around the 17-kDa region with low signals, which suggested the proteolysis of 53 Asp-54 Gln (Fig. 5C). However, the fragment of this sequence was not detected by mAb 21. HRP-labeled anti-IgM Ab detected type 2 as well as IgM under reducing and nonreducing conditions (Fig. 5D). The most likely explanation is that the mAb 21-recognizable 24-kDa IL-18 and unrecognizable 17-kDa fragment bind IgM via disulfide bond at least after purification and storage of type 2 IL-18.

N-terminal amino acid analysis of these proteins, 800 kDa, 70 kDa, and 27 kDa, supported the hypothesis that IgM is the protein responsible for binding the IL-18 moieties, and this form of IL-18 contains novel epitopes neither shared with IL-18 type 1 nor recognized by the conventional mAbs against active rIL-18 (Fig. 5). Only the mAbs produced in this study recognized these novel epitopes of IL-18 type 2 under nonreducing conditions.

To further confirm the presence of the IgM-IL-18 type 2 complex, ELISA was performed in combination with type 2 IL-18-re.recognizable mAbs and anti-human IgM Ab (Fig. 6). The results again supported the presence of the complex.

Discussion

Here, we demonstrated that an IgM-bound form of IL-18 was present in human blood plasma. The active form of IL-18 is a 17-kDa protein (1), here designated type 1, whereas this form of IL-18, named type 2, is of the 17-kDa protein and 24-kDa protein presumably a proform (5). The former may be a proteolytic product of the latter. Our ELISA with inactive rIL-18-specific mAbs (19), but not the conventional ELISA (20), detected this novel IL-18 isoform, suggesting that some structural properties including...
the conformation of type 2 differs from those of type 1 enough to be discriminated by the mAbs. IL-18 type 1 was detected in all volunteers, whereas type 2 was detected in 30% of healthy subjects. The levels of type 2 were high (25–100 ng/ml) compared with those of type 1 (0.05–0.3 ng/ml) in their blood plasma. Strikingly, 24/48-kDa forms of IL-18 were recognized by our mAbs in Mf/Langerhans cells (17) in addition to finding type 2 in blood plasma. Hence, alternative forms of IL-18 should be naturally present in both innate immune competent cells and body fluids. The ratio of IgM:IL-18 in the complex is a point to be settled. The sequences of IL-18 protein of 24 kDa and 17 kDa were minimally detected even under the conditions where the sufficient IgM sequence was identified in the two cases examined, suggesting the low amounts of IL-18 type 2 being complexed with IgM. Pentameric IgM may recruit, 1 molecule of IL-18 type 2. Furthermore, proteolytic cleavage of the 24-kDa proform to the 17-kDa form may proceed either in the blood circulation or during purification. Failure of the reduced 17-kDa fragment to detect by mAb 21 still remains unexplained.

Human IL-18 and IL-12 synergistically act on lymphocytes to induce IFN-γ (7, 9). IL-18 also enhances Fas ligand- and perforin-mediated NK cytolytic activities independent of IL-12 (27). IL-18 serves as an inhibitor of angiogenesis in tumor-bearing mice, the function of which also is in part independent of IL-12 (28, 29). However, we could verify neither the IFN-γ-inducing activity of IL-18 type 2 on NK and T cells nor its binding to IL-18R-positive cells. Again, it barely potentiates NK-mediated cytolytic activity. Thus, the actual function or role of IL-18 type 2 currently remains unknown.

A number of cytokines have been reported to form complexes with plasma proteins. That is, IL-6 covalently binds α2-macroglobulin (14). IL-1β, which belongs to the same superfamily as IL-18 (4), forms high molecular complexes with serum proteins (12). IL-1β also binds α2-macroglobulin and complement C3 via thiol-disulfide exchange reaction (13). IL-2 may covalently bind α2-macroglobulin to be functionally stabilized (15). It is currently accepted that these partners carry the cytokines to the target cells and tissues without leak from the glomerular apparatus to facilitate efficient transportation of cytokine molecules and confer resistance to plasma proteases on cytokine molecules (14, 30). It is of interest how IL-18 type 2 covalently binds IgM. The IgM-containing heterodimeric form would stabilize this IL-18 type 2 molecule to

**FIGURE 5.** A representative immunoblotting profile of IL-18 type 2 and results of N-terminal amino acid analysis. A, Protein staining of purified IL-18 type 2. Five micrograms of the purified sample (see Fig. 4 for purification procedure) were applied to SDS-PAGE followed by Coomassie blue-staining. The amino acid sequences of bands 1, 2, 3, 4, and 5 are indicated in C. B, Immunoblotting profiles of purified IL-18 type 2. The same sample shown in A was blotted onto nitrocellulose sheet and probed with the indicated mAbs directly conjugated to HRP. Left, Nonreduced samples. Right, Reduced samples. The arrow indicate the 24-kDa band. C, Results of N-terminal amino acid analysis. The results were deduced from three independent analyses. Parentheses show the sequence other than those of μ- and κ-chains. The amino acid peaks of this third sequence were significant but minimal compared with those of μ- and κ-chains. Accession numbers of the data from GenBank are shown in the figure. *, Only the most predominant sequence is shown. +, Ser19 appears as Arg/Thr in the database. The proteolytic site for generation of the 17-kDa fragment as well as the caspase-1-cleaving site to yield active IL-18 is shown by arrowheads, which can be deduced from this analysis. D, Comparison of IL-18 type 2 with IgM on blotting. Purified human IgM and IL-18 type 2 were analyzed on SDS-PAGE followed by immunoblotting with anti-IgM μ-chain Ab as a probe. Major bands in the sheets are indicated by open (nonreduced) and filled (reduced) arrowheads. Faster mobility bands in the reduced IL-18 type 2 were not reproducibly observed, suggestive of degradation products of IgM.
survive in blood plasma. Alternatively, IgM attacks foreign material to amplify host defense responses, including C activation (31). Anaphylatoxins are liberated from the C system and recruit Mφ/DC to the inflammatory lesion (31). We favor the interpretation that IL-18 type 2 might have some advantages via conjunction with IgM to express its functions toward the host immune system.

The current concept is that IL-18 is a new member of the IL-1 family on the basis of primary structure, three-dimensional structure, receptor family, signal transduction pathways, and biological effects (4). In fact, studies of IL-18 have been developed with reference to the reported properties of IL-1β (2, 4, 7). However, comprehensive differences exist between IL-1β and IL-18 on their properties: 1) regarding IL-1β, no report speculates the complex formation with IgM or secretion of proform in natural body fluids; 2) comparative studies between IL-1β and IL-18 have indicated...
that caspase-1 activates both IL-1β and IL-18 in a different kinetics in Mφ cytoplasm (5), yet the proform of IL-18 are more competent to secretion from the cells than that of IL-1β; 3) furthermore, IL-18 can be activated via a Fas ligand-mediated and probably caspase-1-independent pathway (32); 4) in vitro studies suggest that a variety of proteases cleaved proIL-1β and proIL-18 into propieces and active form-like fragments (4, 5, 33), and IL-1β may not share at least some of these processing manners or enzymes with IL-18; and 5) thiol-disulfide exchange reaction induces conformational alteration in relevant proteins (34–36). Although the tertiary structures of IL-1β and IL-18 are similar (2, 4), the locations of their five cysteine residues are not always conserved. IL-18, but not IL-1β, may preferentially elicit thiol-disulfide exchange reaction with IgM. Although the mechanism of specific interaction of IL-18 type 2 with IgM will need to be further investigated, IL-18 appears to possess unique properties distinct from IL-1β. The functional properties of IL-18 type 2 and their relationship to diseases remain to be defined. Although premature monocytes failed to produce IL-18 proteins (26), activated Mφ accumulate these isoforms in cytoplasm, which may play some activation-dependent roles in Ag-presenting cells in innate immune activation. IL-18 type 2 has no ability to bind IL-18R complex. In fact, neuroblastoma cells induce a strong and immediate antitumor immune response. IL-18 type 2 has no ability to bind IL-18R complex. In fact, neuroblastoma cells induce a strong and immediate antitumor immune response.

**FIGURE 8.** Binding of IL-18 type 2 to IL-18R-positive cells. Human IgM (top) or purified IL-18 type 2 (25 ng/mL; center) was incubated with L428 (left) or THP-1 cells (right) for 40 min at 4°C. The IL-18-negative IgM preparation (top) was used as a control. After three washes, cells were treated with anti-IgM Ab (top and center), and FITC-labeled second Ab. Cells were analyzed by flow cytometry for detection of the binding of IL-18 type 2 to the IL-18R-positive cells (which should be reflected as dominant shifts of the peaks in the center panel compared with those in the top panel). The levels of IL-18R are shown in the bottom panel.

molecular properties of IL-18 and physiological significance will be clarified in the future.

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**References**


