apoptotic or anti-fibrotic factor as well as an agent stimulating hepatocyte proliferation, administration of recombinant HGF is considered to not only induce liver regeneration, but also inhibit disease progression and ameliorate liver cirrhosis. In the present study, we investigated the pharmacokinetics of recombinant human HGF administered intravenously or via the portal vein. We show here that intravenous injection of recombinant human HGF in a bolus induced an increase in the serum levels of human HGF, and that intravenously administered HGF was primarily distributed to the liver. Conversely, compared with intravenous administration, portal vein injection of recombinant human HGF increased hepatic distribution of the protein, whereas serum HGF levels were reduced. We also demonstrate that, despite the short half-life, the single intravenous injection of recombinant HGF induced tyrosine phosphorylation of c-Met in liver tissues.

2. Materials and methods

2.1. Animals

Eight-week-old male Wistar rats were obtained from Japan SLC Inc. (Shizuoka, Japan). The animals were maintained under constant room temperature (25 °C) and given free access to water and the indicated diet throughout the study. The protocol for animal studies was approved by the ethical committee of the Graduate School of Medicine, Kyoto University (Kyoto, Japan). All animal experiments were performed after a 1-week acclimation period on a standard diet.

Seventy percent partial hepatectomy was performed according to a modification of the method of Higgins and Anderson [24]. The rats were anesthetized with ether and a two-thirds partial hepatectomy was performed. To induce liver fibrosis, the rats were fed a choline-deficient, L-amino acid-defined (CDAA) diet (Dyets Inc., Bethlehem, PA) for 30 weeks. The development of cirrhosis was confirmed by macroscopic inspection and histological examination [25].

2.2. Measurement of serum human HGF

A silicone-rubber catheter (0.5 mm × 1.0 mm o.d.) was inserted into the jugular vein and saline was administered continuously via the catheter using an infusion pump (0.1 ml/h) to prevent obstruction. Recombinant human HGF (0.1 mg/kg) was injected into inguinal vein or splenic vein in less than 10 s, and sequential blood samples were obtained via the catheter 5, 10, 20, 30, 60, 90 and 120 min after the injection.

2.3. Preparation of tissue extracts

Tissue extracts were prepared as previously described [26] with a slight modification. The various tissues were excised 5 min after the intravenous or intraportal injection of recombinant human HGF (0.1, 0.03 or 0.01 mg/kg), and the wet weight of tissue samples was determined. Fresh tissues were homogenized in cold Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (PBS) containing 0.4% EDTA-2Na and 500 units/ml of aprotinin. The homogenates were centrifuged at 9000 × *g* for 20 min at 4 °C, and then at 105,000 × *g* for 1 h at 4 °C. The supernatants were used for measurement of HGF levels and Western blot analysis.

2.4. Measurement of HGF in sera and tissue extracts

HGF levels in serum and tissue extracts were determined by a commercially available ELISA kit (Otsuka Pharmaceutical Co., Tokushima, Japan), in which only human HGF, but not rat HGF, is detected [22,26].

2.5. Western blotting

Tyrosine phosphorylation of c-Met was evaluated by Western blotting. Liver tissues were solubilized in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1 mM Na_3Vo_4, 1 mM DTT, 1 mM PMSF, and 10 μg/ml each of leupeptin, aprotinin, and pepstatin A. Post-nuclear supernatants were precleared with protein A-agarose and immunoprecipitated with anti-c-Met antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and protein A-agarose. Immunoprecipitated materials were washed five times with 0.1% NP40 and 0.05% sodium deoxycholate and eluted by boiling in Laemmli sample buffer (Bio-Rad, Hercules, CA). Samples were separated by 8% SDS-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. After blocking membranes with 1% bovine serum albumin, filters were incubated with horseradish peroxidase-conjugated anti-phosphotyrosine antibody and subjected to ECL Western blotting detection (Amersham Life Sciences, Buckinghamshire, England).

3. Results

3.1. Changes in serum levels of recombinant human HGF in normal, hepatectomized, and cirrhosis rats

We examined changes in serum levels of human HGF following bolus injection of recombinant human HGF using ELISA (Fig. 1). Our ELISA could not detected endogenous rat HGF. When recombinant human HGF (0.1 mg/kg) was injected into normal rats via inguinal veins in less than 10 s, the level of serum human HGF increased to 89.7 ± 20.6 ng/ml 5 min after the injection (Fig. 1A). Recombinant human HGF disappeared from serum with a half-life of 2.4 min, and the serum HGF decreased to 0.65 ± 0.13 ng/ml after 120 min. These findings indicate that the bolus injection of recombinant HGF induced a considerable increase in serum human HGF, followed by disappearance with a short half-life.

Recombinant human HGF will soon be administered to patients with fatal liver disease, including small-for-size grafts

in LDLT or liver cirrhosis. We therefore investigated the effect of loss of liver volume or development of cirrhosis on the serum levels of recombinant human HGF. When recombinant human HGF (0.1 mg/kg) was injected intravenously into rats with 70% partial hepatectomy, the serum levels of human HGF increased to 341.2 ± 55.1 ng/ml at 5 min (Fig. 1A). A significant increase in serum HGF levels was also observed at 10, 30 and 120 min, and the half-life was prolonged (in comparison to normal rats) to 3.8 min. Similarly, the bolus injection of recombinant human HGF into rats with liver cirrhosis led to significantly elevated levels of serum human HGF at each time point and a prolonged half-life (4.4 min).

Conversely, when recombinant human HGF (0.1 mg/kg) was injected to portal veins in normal rats, the serum level of human HGF was only 17.8 ± 5.94 ng/ml 5 min after the injection (Fig. 1B). This is lower than after the intravenous injection; furthermore, the recombinant human HGF injected via portal vein was rapidly decreased with a half-life of 3.0 min (Fig. 1B). In comparison with normal rats, those with 70% partial hepatectomy exhibited significantly higher

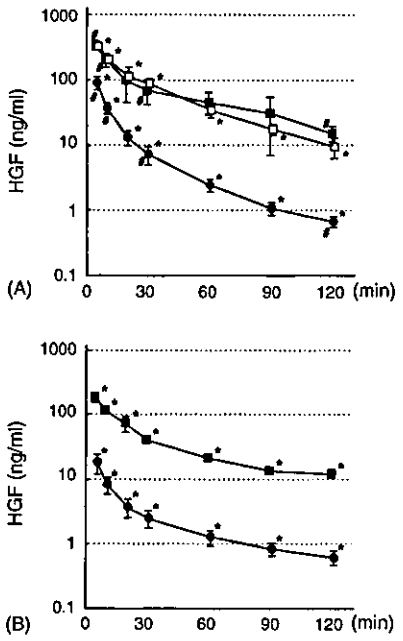

Fig. 1. Serum levels of recombinant human HGF following bolus injection in normal, hepatectomized, and cirrhotic rats. (A) Recombinant human HGF (0.1 mg/kg) was administered intravenously to normal (●: $n = 4$), partially hepatectomized (■: : $n = 3$–4), and cirrhotic (□: $n = 4$) rats, and serum human HGF was determined 5, 10, 20, 30, 60, 90 and 120 min by ELISA as described in Section 2. The serum levels of human HGF at 5 min and its half-life were 89.7 ± 20.6 ng/ml and 2.4 min in normal rats, 341.2 ± 55.1 ng/ml and 3.8 min in rats with partial hepatectomy, and 319.8 ± 22.3 ng/ml and 4.4 min in rats with CDAA diet-induced liver cirrhosis. * and #, $P < 0.05$. (B) Recombinant human HGF was injected via the portal vein in normal (●: $n = 4$) and partially hepatectomized (■: : $n = 3$–4) rats. The serum level of human HGF at 5 min and its half-life were 17.8 ± 5.9 ng/ml and 3.0 min in normal rats and 180.5 ± 29.6 ng/ml and 5.3 min in rats with partial hepatectomy. *, $P < 0.05$.

(180.5 ± 29.6 ng/ml at 5 min) levels of serum HGF following portal vein injection of the recombinant protein, and the recombinant protein had a prolonged half-life (5.3 min). These results suggest that the clearance of recombinant human HGF is dependent on liver volume or the number of hepatocytes, and suggest that the recombinant human HGF is trapped primarily in the liver.

3.2. Tissue distribution of recombinant human HGF administered intravenously or via portal vein

To address whether the liver is the primary organ targeted by recombinant human HGF, we examined tissue distribution of recombinant human HGF administered intravenously or via portal vein. Various tissue samples were obtained 5 min after the bolus injection of recombinant human HGF, and the HGF content of the tissues was measured by ELISA. When recombinant human HGF was administered intravenously, the liver, spleen, adrenal gland, and kidneys contained large amounts of human HGF (290.2 ± 38.3, 582.2 ± 205.0, 278.1 ± 114.1 and 101.3 ± 19.3 ng/g wet tissue, respectively) (Fig. 2, open columns). There were small amounts of human HGF in the lungs, pancreas, and large intestine (16.2 ± 3.2, 6.2 ± 0.9, and 2.0 ± 0.2 ng/g wet tissue, respectively). Although the HGF level per tissue weight in spleen was higher than that in liver, we consider human HGF administered intravenously to be primarily distributed to the liver because the weight of the liver is approximately 20-fold larger than that of the spleen. In contrast, when recombinant human HGF was injected via the portal vein, HGF content in the liver tissues increased to 519.5 ± 218.0 ng/g wet tissue (Fig. 2, closed columns). In comparison with intravenous injection, human HGF levels in extra-hepatic organs, including spleen, adrenal gland, kidney, and lungs, were significantly reduced. These results indicate that the liver is the organ predominantly targeted by intravenously administered recombinant human HGF, and the possibility that portal vein injection of the recombinant protein reduces the effects of HGF on extra-hepatic organs.

3.3. Intravenously injected recombinant human HGF effectively induces c-Met tyrosine phosphorylation

Recombinant human HGF, intravenously administered in a bolus, disappeared from serum with a short half-life. Therefore, we investigated whether a single bolus injection of recombinant human HGF induced tyrosine phosphorylation of c-Met, a specific receptor for HGF, in liver tissues (Fig. 3). Various amounts of recombinant human HGF (0.03, 0.1, 0.3 and 1.0 mg/kg) were injected intravenously in less than 10 s. When 0.1 mg/kg or greater recombinant HGF was administered, c-Met tyrosine phosphorylation was induced 30 min after the injection, and expression of c-Met decreased in parallel with the dose of recombinant HGF. Tyrosine phosphorylation of c-Met was close to background in rats given 0.03 mg/kg of recombinant HGF.

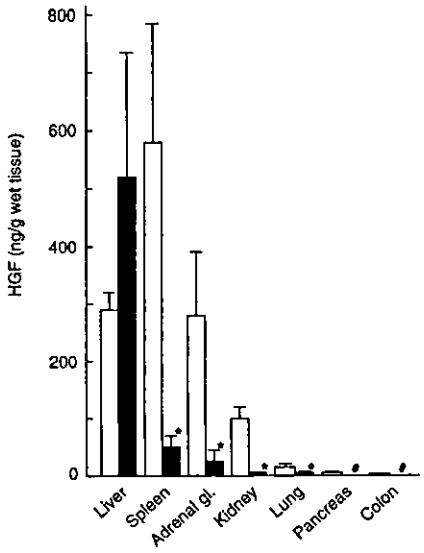

Fig. 2. Tissue distribution of recombinant human HGF administered intravenously or via the portal vein. Various tissue samples were obtained 5 min after bolus injection of recombinant human HGF (0.1 mg/kg). Tissue extracts were prepared and human HGF contents were measured by ELISA as described in Section 2. When recombinant human HGF was injected intravenously, a large amount of human HGF was observed in the liver, spleen, adrenal gland, and kidney (290.2 ± 38.3, 582.2 ± 205.0, 278.1 ± 114.1 and 101.3 ± 19.3 ng/g wet tissue, respectively). The amount was smaller in lung, pancreas, and large intestine (16.2 ± 3.2, 6.2 ± 0.9 and 2.0 ± 0.2 ng/g wet tissue, respectively) (open columns) ($n = 4$). Bolus injection via portal vein significantly reduced the distribution of this protein to extra-hepatic organs (spleen, adrenal gland, kidney, and lung), while HGF content in liver tissues was increased to 519.5 ± 218.0 ng/g wet tissue (closed columns) ($n = 4$). #, HGF content in pancreas and large intestine of rats that received human HGF via the portal vein were not determined. *, $P < 0.05$ in comparison with HGF content in each organ of rats treated intravenously with recombinant human HGF.

3.4. Rats with partial hepatectomy, given recombinant human HGF via the portal vein, exhibited reduced extra-hepatic distribution and increased HGF content in liver tissues

Administration of recombinant human HGF via portal vein is suitable for patients with partial hepatectomy or recipients of LDLT. We therefore investigated the effect of 70% partial hepatectomy on HGF levels in the liver, spleen, and kidneys 5 min after the portal vein injection (Fig. 4). When 0.1 mg/kg HGF was injected via the portal vein in hepatectomized rats, the HGF content in liver, spleen, and kidneys increased to 1262.0 ± 616.6, 82.0 ± 18.3 and 38.5 ± 5.8 ng/g wet tissue, respectively, which is relatively higher than in normal rats (Fig. 2). Administration of a lower dose of recombinant HGF resulted in lower HGF content in liver, spleen, and kidneys. Interestingly, when 0.03 mg/kg of HGF was administered to 70% partial hepatectomized rats via the portal vein, the HGF content in liver tissues (547.8 ± 153.3 ng/g wet tissue) was approximately the same as that in normal rats administered with 0.1 mg/kg of the protein (Figs. 2 and 4). The

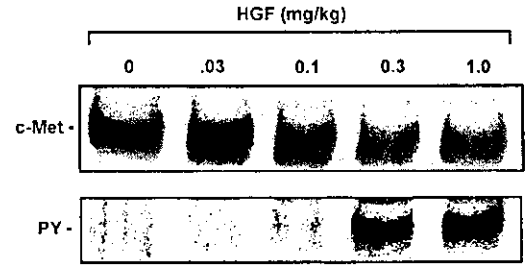

Fig. 3. Bolus injection of recombinant human HGF induced c-Met tyrosine phosphorylation in liver tissue. Recombinant human HGF (0.03, 0.1, 0.3 and 1.0 mg/kg) or PBS was intravenously administered to normal rats. Liver tissues were obtained 30 min after bolus injection. Expression and tyrosine phosphorylation of c-Met were examined by Western blotting, as described in Section 2. Administration of more than 0.1 mg/kg of recombinant human HGF induced c-Met tyrosine phosphorylation in liver tissue, and expression of c-Met decreased in parallel with the dose of recombinant HGF.

dose of recombinant human HGF could be reduced to 30% to treat hepatectomized animals with a remnant liver volume of 30%. These results indicate that when recombinant human HGF is administered to patients with partial hepatectomy or recipients of LDLT, the protein dose should be modulated according to the remnant liver volume.

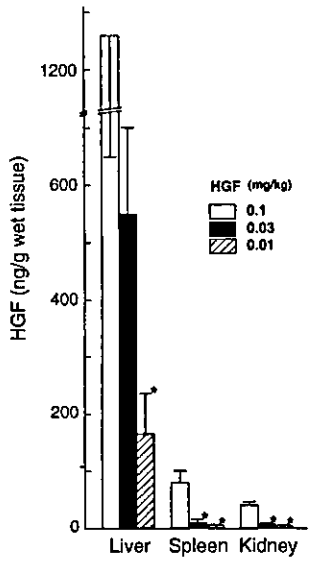

Fig. 4. Tissue distribution of recombinant human HGF administered via the portal vein to rats with 70% partial hepatectomy. Various doses of recombinant human HGF (0.01, 0.03 and 0.1 mg/kg) were administered to rats via the portal vein following 70% partial hepatectomy. The liver, spleen, and kidneys were obtained 5 min after injection, and tissue content of human HGF was measured by ELISA, as described in Section 2. Hepatectomized rats given 0.1 mg/kg of recombinant HGF exhibited an increase in hepatic and extra-hepatic distribution of the protein. When 70% hepatectomized rats were treated with 0.03 mg/kg of recombinant human HGF, its content in liver tissues (547.8 ± 153.3 ng/g wet tissue) was approximately the same as in normal rats given 0.1 mg/kg of recombinant HGF, as shown in Fig. 2. *, $P < 0.05$ in comparison with the HGF content in each organ of hepatectomized rats given recombinant human HGF (0.1 mg/kg).

4. Discussion

HGF is produced from mesenchymal cells as an inactive precursor, pro-HGF [27–29]. After tissue injury, pro-HGF is converted to an active heterodimer, consisting of light and heavy chains, primarily by a specific serine protease, HGF activator (HGFA) [30–32] as well as weakly by blood-coagulation factor XIIa [33]. Recently, HGFA inhibitor (HAI)-1, which was first identified as an inhibitor of HGFA [34], has been reported to act as a reservoir for HGFA in injured tissues [35]. Thus, HGF may play an important role in regeneration and repair of injured tissues, including the liver and intestine. In the present study, we investigated the pharmacokinetics of recombinant human HGF, the heterodimeric active form of HGF. Bolus injection of recombinant human HGF led to its distribution primarily to the liver and effectively induced c-Met tyrosine phosphorylation in liver. Therefore, despite its short half-life, it is possible that intravenously administered recombinant human HGF induces liver regeneration in patients with fatal liver disease. Indeed, intravenous bolus injection of recombinant HGF led to a considerable increase in serum HGF levels (Fig. 1). Treatment with this protein increased liver weight and serum albumin levels in a rat model of dimetylnitrosamine-induced liver injury, in which serum levels of endogenous rat HGF were increased to 2–3 ng/ml, (unpublished data), as well as following partial hepatectomy [36,37]. In addition, a single intravenous injection of recombinant human HGF rescued mice from anti-CD95-induced lethal hepatic failure via its anti-apoptotic effects (unpublished data), and HGF-induced inhibition of apoptosis has been reported in various models of hepatic injury, using HGF gene transfer or HGF protein [9–15]. Thus, when recombinant human HGF is administered to patients with fulminant hepatic failure, the treatment is thought to not only stimulate liver regeneration, but also inhibit disease progression through its anti-apoptotic activity.

When recombinant human HGF was administered intravenously to rats with 70% partial hepatectomy or CDAA diet-induced cirrhosis, serum levels of human HGF were elevated. These results suggest that extra-hepatic distribution of administered HGF is increased in patients with liver disease, leading to adverse side effects. Bolus injection of recombinant HGF actually induced considerable levels of HGF in renal tissue (Fig. 2). However, although HGF has been reported to ameliorate renal fibrosis and dysfunction [16], the repeated injection caused reversible proteinuria (unpublished data). Conversely, bolus injection via the portal vein was capable of delivering recombinant human HGF more specifically to the liver, reducing extra-hepatic distribution of the protein. Nevertheless, even when recombinant human HGF was administered via the portal vein, hepatectomized rats exhibited an increase in HGF levels in the liver, extra-hepatic tissues, and serum. Elevated human HGF contents in the remnant livers may be due to an increase in the amount of administered human HGF per liver volume, and it is possible that, once c-Met receptor and extracellular matrix in liver tissues are saturated, recombinant human HGF leads to an increase in human HGF levels in the serum and extra-hepatic tissues. Therefore, although intraportal injections are thought to reduce the effects on extra-hepatic organs, the dose of recombinant human HGF should be reduced according to the degree of liver injury or remnant liver volume regardless of the route of administration. Indeed, recombinant human HGF administered to rats with 70% partial hepatectomy could be reduced to 30% to achieve the same HGF level in liver tissues as in normal rats (Figs. 2 and 4).

HGF is also known to stimulate liver regeneration and reduce fibrosis in experimental models of liver cirrhosis [17–21], and administration of recombinant human HGF has the potential to become a new modality for cirrhosis patients. However, since lengthy HGF administration is likely required to treat cirrhotic patients, development of an appropriate drug delivery system for liver-specific targeting or long-term release is desirable. Additionally, the potential of HGF to stimulate hepatocellular carcinomas needs to be evaluated before beginning clinical applications.

HGF plays an important role in the repair of injured intestinal mucosa. We have recently reported that administration of recombinant human HGF, using osmotic pumps implanted into peritoneal cavities, resulted in a persistent increase in serum human HGF and facilitated colonic mucosal repair in a rat model of experimental colitis [38]. However, in the present study, the human HGF content of colon tissues was extremely low following intravenous injection of the recombinant protein. Therefore, it is necessary to explore whether repeated injections of human HGF ameliorate intestinal mucosal injury. Alternatively, tissue-specific delivery of the recombinant protein should be established to treat inflammatory bowel disease. Effectiveness of HGF has also been reported in animals with injury of various extra-hepatic organs, including kidney, lung, pancreas, and neurons [16,39–41]. Therefore, development of a delivery system for tissue-specific or long-term release may contribute to expanding the clinical application of recombinant human HGF to diseases of extra-hepatic organs, such as renal sclerosis, pulmonary fibrosis, severe pancreatitis, or neurodegenerative disease.

In conclusion, our findings presented here suggest that although recombinant HGF disappears rapidly from serum, bolus injection of recombinant human HGF may exert a therapeutic effect in patients with fatal liver disease.

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Degenerate recognition and response of human CD4+ Th cell clones: implications for basic and applied immunology

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Abstract

It was once considered that the T cell response is an all or nothing type event, but recent studies have clearly indicated that T cells show many different types of activation in recognition of altered ligands for T cell receptors (TCR). In this review, we summarize our recent findings on the response of human CD4+ helper T (Th) cell clones to altered peptide ligands (APL); peptides carrying single or multiple residue substitutions in antigenic peptides. The extensive analyses revealed that TCR-antagonism and partial agonism are frequently observed by the stimulation with APLs substituted at particular amino acid residues of antigenic peptides. We observed unique partially agonistic APLs inducing prolongation of T cell survival without cell proliferation. Superagonistic APLs stimulated enhanced proliferation and production of cytokines in Th cell clones reactive to tumor-associated antigens. The other APL induced enhanced production of interleukin-12 by antigen presenting cells and subsequent enhancement of IFN-γ production by T cells reactive to allergens. By utilizing an HLA-DR-restricted T cell epitope library generated by mutated invariant chain genes, it was revealed that human Th cell clones recognize a more diverse array of peptides with multiple and simultaneous amino acid substitutions in an antigenic peptide. APLs also induced altered intracellular signaling events including intracellular calcium increase and phosphorylation of signaling molecules. This information provides basic knowledge regarding the characteristics of antigen recognition by human Th cells and the subsequent activation, and a novel method for manipulation of human Th cell responses by APLs, as a possible candidate for antigen-specific immuno-potentiating or immunosuppressive therapy.

Keywords: HLA class II molecule; Human CD4+ Th-cells; Antigenic peptides; Altered peptide ligands; T cell recognition; TCR antagonism; Peptide partial agonism

1. Introduction

The human histocompatibility leukocyte antigen class-II (HLA-II) molecule has a peptide binding groove on top of the molecule and binds antigenic peptides processed by antigen presenting cell (APC) to present them to CD4+ helper T (Th) cells (Germain and Margulies, 1993). Three to five amino acid residues were separated by one to two intervening residue(s) and acted as anchor residue(s) for peptide binding to HLA-II molecules (Sette et al., 1993; Hammer et al., 1993; Matsushita et al., 1994). On the other hand, side chains of amino acid residues flanking anchor residues proved to be the main recognition sites by T cell receptors (TCR); this was clearly established in crystallographic analyses of the DR molecule bound by either self (Brown et al., 1993) or non-self peptides (Stern et al., 1994).

CD4+ Th cells usually recognize non-self peptides in the context of self HLA-DR molecules. Recognition and responses of T cells were once considered to be an on/off phenomenon, however recent findings obtained using altered peptide ligands (APLs) carrying single residue substitutions in antigenic peptides presented by one major histocompatibility complex (MHC) class II molecule or one specific peptide presented by different MHC class II molecules showing a limited polymorphism revealed that

Abbreviations: HLA-II, human histocompatibility class II; APC, antigen presenting cell; Th cell, helper T cell; TCR, T cell receptor; APL, altered peptide ligand; MHC, major histocompatibility complex; IFN-γ, gamma interferon; IL, interleukin; CLIP, class II-associated invariant chain peptide; ZAP-70, zeta-associated protein-70

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altered TCR ligands induce altered T cell responses in both mice and humans, including (1) T cell non-responsiveness, through TCR antagonism and (2) partial agonism inducing partial activation of T cells without cell proliferation (Sloan-Lancaster and Allen, 1996).

Previous analyses also revealed that the interactions of TCRs with MHC-wild-type peptides had stronger affinities and/or smaller off-rates than did those of TCRs with MHC-APL complexes (Lyons et al., 1996). These differences in characteristics of molecular interactions may induce insufficient engagements of TCR with MHC-APL complexes such that intracellular signals mediated by TCR through recognition of APLs are inadequate for full activation of T cells to induce cell proliferation. In some cases, inadequate signals induce unique altered T cell responses.

In this review, we will summarize our recent analyses on recognition by human $CD4^+$ Th cell clones of diverse peptides, and the heterogeneity of subsequent T cell responses and T cell activation signals induced, as summarized in Table 1.

2. Frequencies of agonistic and antagonistic single residue substituted APLs depend on position of substituted amino acid residues of the peptide

If there is a general rule for structures of APLs which stimulate or inhibit T cell responses to wild type antigenic peptides, it would be easier to generate peptides which augment or inhibit responses of human Th cells. We used a human Th1-cell like clone YN5-32 reactive to a streptococcal M12p54-68 peptide (^{54}NRDLEQAYNELSGEA^{68}) in the context of HLA-DR4 (DRB1*0406), and analyzed responses of YN5-32 to 156 independent APLs carrying single residue substitutions at residues 57 (P1)-65 (P9) of the peptide where P1 (position 1) means the putative most N-terminal DR anchor residue (Chen et al., 1996). As shown in Fig. 1, residues Leu-57 (P1), Ala-60 (P4) and Asn-62 (P6) were the most likely to be DR-anchor residues, and 30% (17/57) of APLs substituted at these residues exhibited full agonism to stimulate various magnitudes of proliferative responses in the T cell clone, whereas only 7.5% (3/40) of non-fully agonistic peptides exhibited TCR antagonism. On the other hand,

Table 1
Summary of our observations on responses of human $CD4^+$ Th-cell clones to APLs

Th-cell clone	Specificity	Observed immune responses to APLs	Reference
YN5-32	Streptococcal M12p54-68/DR4	TCR antagonism	
		Partial agonism; increases in cell size and expression levels of CD4, 11a, 28, 49d, 95 without anergy induction	Chen et al. (1996)
		Polymorphism at DRβ37 affected T cell recognition	Chen et al. (1997)
		Quantitative and qualitative alteration of intracellular calcium increase	Chen et al. (1998)
		Overexpression of partially agonistic TCR-ligand induced proliferation without phosphorylation of ZAP-70 and LAT	Irie et al. (2003)
SK2.11	AChRα p75-87/DQ6	TCR antagonism	Kanai et al. (1997).
BC20.7, BC33.5, BC42.1	BCGa p84-100/DR14	Partial agonism in recognition of artificial or natural self APLs; increased survival without antigenic stimuli or production of IL-4 and IFN-γ without cell proliferation	Matsushita et al. (1997)
C27	p21Ras p3-17/DR1	Superagonism; increased proliferation and production of IFN-γ and GM-CSF in recognition of cancer-associated mutated peptides and its APL	Yokomizo et al. (1997)
Y41.2	TEL/AML1 fusion peptide/DP17	Superagonism; increased proliferation and production of IFN-γ and GM-CSF in recodnition of APLs derived from leukemia-associated TEL/AML1 fusion peptide	Yun et al. (1999)
29.15.2	p21Ras p3-20/DR51	Superagonism; increased proliferation in recognition of APL identified by using a combinatorial peptide library and mass spectrometry	Tanaka et al. (1999)
ST1.9	*Cry j*1p335-346/DR52	Superagonism; increased production of IFN-γ	Ikagawa et al. (1996)
DT13.2	*Der f*1p18-31/DQ6	Superagonism; increased production of IFN-γ stimulated by increased production of IL-12 from antigen presenting cells	Matsuoka et al. (1996)
SA32.5, MK20.2	GAD65 p115-127/DR53	Generation of a multiple residue substituted epitope expression library by using CLIP-substituted invariant chain genes to identify agonistic APLs and mimicry microbial peptides	Uemura et al. (2003)

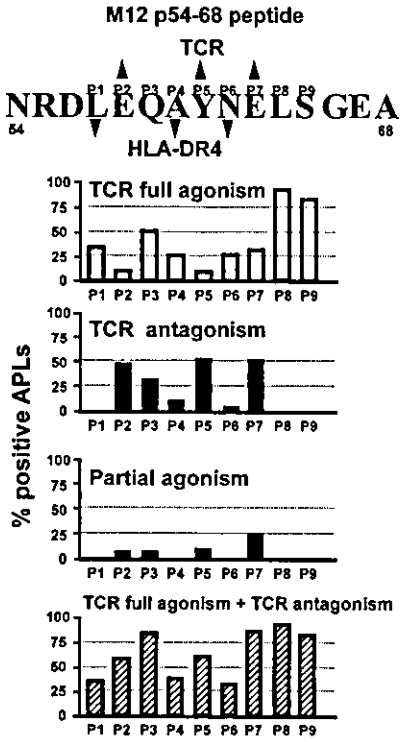

Fig. 1. Summary of responses of the human Th cell clone YN5-32 to 156 APLs carrying single residue substitutions in a streptococcal antigenic peptide M12p54-68. From P1 to P7 residues, residues were replaced with 19 other amino acids. The P8 and P9 residues were replaced with 10 and 11 other amino acids, respectively. Percentages of APLs exhibiting either full agonism (open bars), TCR antagonism (shedded bars) or partial agonism (closed bars) are indicated for each residue. APLs carrying substitutions at putative TCR contact residues, P2, P5 and P7, frequently exhibited TCR antagonism. Some of them, especially APLs substituted at P7, exhibited partial agonism. Because APLs with full agonism or TCR antagonism have to bind to MHC molecules, the frequencies of those peptides indicated by cross hatched bars represent the frequency of peptides with MHC-binding capacity.

residues Glu-58 (P2), Tyr-61 (P5) and Glu-63 (P7) were the most likely to be TCR-recognition sites and only 15.8% (9/57) of APLs stimulated proliferative responses in YN5-32 thereby indicating that substitutions at these residues frequently abrogate T cell recognition. Interestingly, as many as 60.4% (29/48) of non-fully agonistic APLs exhibited TCR antagonism to inhibit the proliferative response of YN5-32 to the wild-type peptide.

Eight (27.6%) of these antagonistic APLs carrying relatively conservative amino acid substitutions exhibited partial agonism to induce large increases in cell size and expression levels of CD4, CD11a (LFA-1α), CD28, CD49d (VLA-4α) and CD95 (Fas), on the T cell surface, as compared with responses to the wild-type peptide. This was the most prominent at Glu-63 (p7) where 5 of 10 antagonistic APLs exhibited partial agonism. These observations indicate that many APLs carrying substitutions at TCR recognition sites in the T cell epitope induce a partial agonism as well as TCR antagonism in YN5-32, as noted by other studies of mouse T cell clones. Differences, such as the absence of anergy induction or little increase in CD25 expression by partially agonistic APLs have been noted in human Th cells. The polymorphism (Ser-Tyr) at the DRβ^{37} residue induced conformational changes of peptides, which can be distinguished by YN5-32 TCR in some but not all peptides, providing further evidence for altered human T cell responses induced by minor modifications of TCR ligands (Chen et al., 1997).

Based on this knowledge of Th cell responses to APLs, we identified many antagonistic APLs which can inhibit proliferation of Th-cell clones auto-reactive to the acetylcholine receptor α chain derived self peptide in the context of the disease-susceptible HLA-DQ6 molecule and established from a patient with infant-onset myasthenia gravis unique to Asian populations (Kanai et al., 1997).

3. Unique partially agonistic APLs inducing prolonged survival of Th cells in the absence of antigenic stimulus

By utilizing three other human Th cell clones with distinct TCR-Vβ recognizing the same non-self mycobacterial *Bacillus Calmette-Guérin* a (BCGa) peptide/HLA-DR14 complex, we found another type of unique partial agonism, as follows (Matsushita et al., 1997). Stimulation of T cells with a one-residue-substituted APL or a minimally homologous self-peptide fragment can prolong the in vitro survival of T cells in the absence of antigenic stimuli, in a clone specific-manner. This prolongation is associated with the up-regulation of Bcl-x_L, without proliferation and these peptide-clone combinations are capable of inducing lymphokine secretion. Thus, peptide partial agonism may play a role in the survival of not only thymocytes but also mature Th cells, in the absence of non-self peptide ligands.

4. Augmentation of T cell responses (superagonism) stimulated by APLs: implication to peptide-based cancer immuno-therapy

A T cell response to a tumor requires a tumor antigen processed into peptides which can be presented to $CD8^+$ cytotoxic T cells by MHC class I molecules, and to $CD4^+$ Th cells by MHC class II molecules. While cytotoxic T cells can kill tumor cells directly, some Th1 cells can mediate cytotoxicity to tumors, amplify responses of cytotoxic T cells, and activate APC, through secretion of lymphokines to augment anti-cancer immunity. We established a Th cell clone reactive to oncogenic and mutated p21 Ras proteins as well as mutated peptides, in an HLA-DR1-restricted manner. We provided evidence for augmentation of proliferation and production of gamma interferon (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-

CSF) by this T cell clone in recognition of APLs carrying a single residue substitution in the mutated P21 Ras peptide (Yokomizo et al., 1997).

We also identified superagonistic and single residue substituted APLs derived from leukemia-associated TEL/AML1 fusion peptide (IGRIA/ECILGMNPSR) (Yun et al., 1999). The APLs having Val or Leu substitutions at putative P8(Gly) or P9(Met) of the peptide respectively stimulated much stronger proliferation and production of Th1-type cytokines in a Th clone reactive to TEL/AML1 fusion peptide in the context of HLA-DP17. These superagonistic APLs can be given consideration for anti-leukemic immunotherapy.

To identify peptide superagonists in a systematic and sophisticated manner, we used a combinatorial peptide library and mass-spectrometry (Tanaka et al., 1999). The proliferative responses of a human $CD4^+$ T cell clone reactive to a self-K-Ras-derived peptide, Ras p3-20 (^{3}EYKLVVVGAGGVGKSALT^{20}), were tested using a set of X9 combinatorial peptide libraries containing the flanking residues (EYKLVXXXXXXXXXSALT, where X indicates random amino acids). Certain peptide libraries, such as EYKLVXXXXXXMXXSALT and EYKLVXXXXXXXHXSALT, stimulated a marked proliferation of T cells. However, no combinations of substitutions tested, such as EYKLVXXXXXXMHXSALT, exhibited additive effects. We subsequently synthesized peptides with degenerate sequences (a mixture of 480 species), where each position is composed of the wild-type residue or of amino acids that induced the proliferation of T cells, in positional scanning. Interestingly, one fraction of degenerate peptides, separated by reverse-phase HPLC, stimulated a much stronger proliferation than did the Ras p3-20; in addition, the retention time of this fraction was distinct from that of Ras p3-20. Mass spectrometry analysis of this fraction and flanking fractions identified five peptide species that exhibit strong signals in a manner that parallels the antigenic activity. Finally, 17 candidate peptide sequences were deduced from mass spectrometry and hydrophobicity scoring results, of which two peptides (EYKLVVVGAGGMLKSALT and EYKLVVVGAGGMIKSALT) did induce 52- and 61-fold stronger proliferation, respectively, compared with the Ras p3-20. These findings indicate that: (1) synthetic peptides that carry "the best" residue substitution at each position of combinatorial peptide libraries do not always exhibit superagonism, and (2) such a drawback can be overcome with the use of mass spectrometry. This approach provides new perspectives for accurate and efficient identification of peptide superagonists.

5. APL affects not only T cell responses but also APC responses to increase IL-12 production: Implication to peptide therapy inducing Th1-dominance

Human Th0 clone DT13.2 reactive to the group I allergen in *Dermatophagoides farinae* extracts (*Der f* I) p18-31 (^{18}RSLRTVTPIRMQGG^{31}) in the context of HLA-DQ6 (DQA1*0102/DQB1*0602) molecules was generated from a patient with bronchial asthma and DT13.2 produced both interleukin (IL)-4 and IFN-γ. Analysis of changes in DT13.2 responses to *Der f* I p18-31-derived APLs revealed that the substitution of ^{27}Arg to Lys resulted in a significant increase in IFN-γ production, with no remarkable changes either in proliferative response or in IL-4 production (Matsuoka et al., 1996). Interestingly, the selective enhancement of IFN-γ by the APL was accompanied by an increased production of IL-12 and this event was suppressed by an anti-IL-12 antibody down to the level of IFN-γ production induced by the wild-type peptide. The superagonistic APL derived from another Japanese Cedar pollen allergen (*Cry J*I) also augmented production of IFN-γ in a human Th0 clone reactive to *Cry J*I peptide/HLA-DR52 complex (Ikagawa et al., 1996).

Our observations suggest that the mode of interaction between TCR and MHC/peptide complex may determine the Th1-predisposing condition by controlling the IL-12 production by APC. Furthermore, this kind of Th1-response inducing APLs may provide peptide therapy for diseases caused by Th2 responses such as allergy.

6. Generation of a Th cell epitope expression library for extensive analysis of degeneracy in peptides recognized by human Th cell clones

Because we found that the systematic detection of cross-recognized epitopes considering the combinatorial effect of amino acids within the epitope is impossible in approaches using positional scanning synthetic combinatorial peptide libraries, we established an alternative method by utilizing molecular genetic approaches. A DNA-based randomized epitope library using class II-associated invariant chain peptide (CLIP)-substituted invariant chains was generated (Fujii et al., 1998; Fujii et al., 2001; Uemura et al., 2003). This approach, by which multiple residues of an antigenic peptide were simultaneously randomized, has the great advantage of producing several conformations of the peptide/HLA-II complexes, and increasing the possibility to identify degenerate sequences with agonistic properties. GAD65-autoreactive T cell clones restricted by disease-susceptible HLA-DR53 and established from patients with type I diabetes were utilized as models. Analysis of agonistic epitopes indicate that recognition by each TCR was significantly affected by combinations of amino acids in the antigenic peptide, although the degree of combinatorial effect differed between each TCR. Protein database searching based on the TCR recognition profile proved successful in identifying several microbial and self-protein-derived mimicry epitopes with limited sequence homology to the original GAD65 epitope. Some of the identified mimicry epitopes were actually produced from recombinant microbial proteins by APCs to stimulate T cell clones. Our data

demonstrate the importance of the combinatorial nature of amino acid residues of epitopes to investigate diversity of T cell recognition and molecular mimicry, and the Th cell epitope display library we established provides a useful tool for these objectives.

7. Altered intracellular signalings induced in a Th-cell clone by APLs

In mouse T cell clones, TCR antagonistic or partially agonistic APLs induce partial phosphorylation of CD3ζ chains leading to the absence of phosphorylation and activation of ZAP-70 (Sloan-Lancaster et al., 1994; Madrenas et al., 1995). Studies of calcium signaling activity in mouse T cells stimulated with APLs indicated that the Ca^{2+} response induced by antagonistic APLs was smaller in amplitude and shorter in duration than that induced by fully agonistic ligands (Sloan-Lancaster et al., 1996; Wülfing et al., 1997).

To determine if APLs affect intracellular activation signals in human Th cells, we investigated changes in intracellular calcium concentrations ($[Ca^{2+}]_i$) in the Th cell clone YN5-32 stimulated with either fully agonistic peptide M12p54-68 or partially agonistic APL E63V (standing for APL having Val-substitution at amino acid residue 63 Glu), or simply antagonistic APL E58M as described in the Section 1 (Chen et al., 1998). Both E63V and E58M stimulated a Ca^{2+} response in ~40% of the T cells, whereas M12p54-68 did so in ~70% of T cells. The most predominant pattern of a Ca^{2+} increase induced by M12p54-68 was a small sinusoidal peak followed by a sustained high response. The most frequent pattern of calcium response induced by E63V was a continuous high response without a preceding sinusoidal peak, whereas that induced by E58M was large with frequent oscillations. Furthermore, our results suggest that the Ca^{2+} response induced by the fully agonistic peptide depends on activation of the genistein-sensitive signaling pathway, including protein tyrosine kinases, whereas the Ca^{2+} response to a simple antagonistic APL completely depends on activation of the GF109203X-sensitive signaling pathway, including protein kinase Cs and extracellular Ca^{2+}. These differences in the $[Ca^{2+}]_i$ response in recognition of different APLs may parallel the unique T cell activation patterns induced by APLs in human T cells.

We then asked whether forced overexpression of partially agonistic TCR-ligands on APCs provides high-avidity TCR-ligands to stimulate T cell proliferation, we generated L cell transfectants expressing various numbers of HLA-DR4 covalently linked with APLs derived from M12p54-68 peptide and observed responses of the cognate T cell clone YN5-32. Some overexpressed HLA-DR4/partially agonistic APL complexes induced T-cell proliferation in a density-dependent manner, however tyrosine-phosphorylation of ZAP-70 and linker for activated T cells (LAT) and kinase activity of ZAP-70 were not detectable (Irie et al., 2003). Our data suggest the presence of an unique signaling pathway coupling TCR-ligation with T cell proliferation in a ZAP-70 less dependent manner, and this activation pathway is observed when TCRs are engaged with relatively low affinity TCR ligands expressed in high density on the surface of APC. This suggests that T cell activation signals are not uniform and they can be alternatively activated depending on binding characteristics between TCRs and their ligands.

8. Conclusions

In conclusion, we observed various kinds of responses to APLs in human Th cell clones, as summarized in Table 1, and the implications of our findings are as follows. (1) It is so far difficult to predict degeneracy of Th cell recognition in a given TCR by analyzing the past literature, and our Th cell epitope expression library using CLIP-substituted invariant chain genes will provide a breakthrough in this field. (2) Our findings may support the following ideas, (1) maintenance of Th cell survival (memory ?) by self APLs in the absence of stimuli with non-self peptides, (2) triggering of autoreactive Th cells by non-self agonistic APLs (molecular mimicry), and (3) a possible application of APLs to augmentation of desirable anti-microbial or anti-tumor immunity, or to inhibition of pathological immune responses such as allergy and autoimmunity. Our analyses of human Th cell responses to APLs have provided pertinent information on the basic immunology of human Th cell biology and also on the strategy for new methods for manipulation of antigen-specific responses of human Th cells.

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Monocytes of distinct clinical types of leprosy are differentially activated by cross-linking class II HLA molecules to secrete IL-12

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Ohyama H, Kato K, Takeuchi K, Soga Y, Uemura Y, Nishimura F, Matsushita S. Monocytes of distinct clinical types of leprosy are differentially activated by cross-linking class II HLA molecules to secrete IL-12. APMIS 2004;112:271–4.

Leprosy is characterized by a wide spectrum of clinical features depending on the individual differences in Th1-type immunity. The objective of this study was to evaluate whether monocyte activation by stimulus via class II HLA molecules would be correlated with the differences in cellular immune responses among diverse clinical forms of leprosy. IL-1β and IL-12 productivity in monocyte preparations obtained from PBMCs was estimated in patients with lepromatous- and tuberculoid-type leprosy. We found that monocytes from lepromatous patients produced significantly higher (about 4-fold higher) amounts of IL-12 as compared to in patients with tuberculoid type of leprosy when class II HLA molecules were cross-linked with anti-HLA class II antibodies, whereas almost equal amounts of IL-1β were produced from each monocyte preparation by stimulus via class II HLA molecules regardless of the clinical form of leprosy. These results suggest that monocyte activation differs between lepromatous and tuberculoid patients in terms of IL-12 secretion, which might be related to individual differences in the cellular immune responses according to the clinical type of leprosy.

Key words: Class II HLA; IL-12; leprosy patients; cellular immune response.

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Leprosy, a chronic disease caused by infection with *Mycobacterium leprae*, shows a wide spectrum of clinical features (1). Tuberculoid type of leprosy is at one end and lepromatous leprosy at the other end of the spectrum. Tuberculoid patients show a high level of cell-mediated immunity (CMI) responses against *M. leprae*, which results in resistance to infection, whereas lepromatous patients show poor CMI responses against the pathogen and progressive form of the disease. This clinical spectrum of leprosy is explained by the differences in responses to *M. leprae* among individuals. The differences of T-cell subsets accumulating in leprosy lesions may account for the diversity of protective patterns to *M. leprae*. In the case of *M. leprae* infection, the clinical type of the disease depends on individual differences in Th1-type immunity. One of the most important cytokines produced by Th1, IFN-γ, promotes activation of macrophages, thus leading the host immunity toward cellular immunity to these bacteria. IL-12, which is produced by antigen-presenting cells (APCs), is

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well-known as a powerful inducer of IFN-γ production from Th1 cells. In leprosy patients, IL-12 productivity is likely to influence the cellular immune responsiveness in patients with lepromatous and tuberculoid type of the disease (2, 3). Toll-like receptor 2 (TLR2) is thought to be a key molecule in inducing the IL-12 production from monocytes in the response against mycobacterial pathogens (4), and the polymorphism of the TLR2 gene is likely to affect the low productivities of IL-12 in the lepromatous type of leprosy patients (5).

Meanwhile, recent studies have suggested that class II HLA (HLA-II) molecules not only act as antigen-presenting molecules, but also as receptor molecules to transduce signals into APCs, resulting in the production of several cytokines, including IL-1β and IL-12 (6). From this point of view, it is likely that the difference in IL-12 production induced by the stimulus via HLA-II molecules will also be a possible factor implicated in determination of the clinical type of leprosy.

In this study, we measured IL-12 production from APCs by stimulus via HLA-II molecules in humans with leprosy, in order to find the differences in CMI activities in vitro between the patients with lepromatous and tuberculoid types of leprosy.

MATERIALS AND METHODS

Study population

Ten leprosy patients, including 5 LL patients and 5 TT patients, and 7 healthy individuals were enrolled in this study. Patients were clinically diagnosed as having each type of leprosy based on the description of Ridley & Jopling (1) and with reference to their results in the Mitsuda test and their sequelae.

Cell preparation

Mononuclear cells were prepared by the Ficoll-paque (Pharmacia Biotech, Uppsala, Sweden) gradient solution method. Peripheral blood mononuclear cells (PBMC) were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated pooled human sera (HS) and placed on HS-pretreated plastic dishes (Falcon, Becton Dickinson, Lincoln Park, NJ). These dishes were incubated for 2 h at 37°C. After the dish-nonadherent cells were harvested and extensive washes, the adherent cells were removed by washing with ice-cold PBS containing 0.04% EDTA and by scraping them with a rubber policeman. This fraction was served as a monocyte fraction. More than 90% of the adherent cell fraction was CD14-positive.

Inducing the monokine production from monocytes

Mouse anti-HLA-DR (L243: Leinco Technologies Inc., Ballwin, MO), anti-DQ (1a3: Leinco Technologies Inc.) monoclonal antibodies (mAbs) and isotype-matched control antibodies (mouse IgG2a) were coated onto 96-well flat-bottomed culture plates for a day at 10 μg/ml PBS. After extensive washing of the plates with PBS, monocytes were added at 2×10^4 cells/well, and incubated for 16 h at 37 °C in a CO_2 incubator (7).

Quantitation of cytokines

Culture supernatants of monocytes stimulated via HLA-II molecules were collected. The hIL-1β and hIL-12 (p40 & p70) ELISA kits (Endogen Inc. Woburn, MA) were used for the quantitation of cytokines in the supernatants. The detection limit of both these cytokines is 5 pg/ml.

RESULTS AND DISCUSSION

We first estimated IL-1β and IL-12 productivity in monocyte preparations obtained from PBMCs of lepromatous, tuberculoid, and healthy Japanese subjects. Monocytes from lepromatous patients produced significantly higher amounts of IL-12 as compared with those from patients with tuberculoid type leprosy (Mann-Whitney *U* test; $p<0.05$), whereas almost equal amounts of IL-1β were produced from each monocyte preparation by stimulus via HLA-II molecules regardless of the clinical form of leprosy. In particular, monocyte preparations from three tuberculoid patients produced extremely low amounts of IL-12. A small amount of IL-β and IL-12 was detected when monocytes were cultured in a dish coated with isotype-matched antibodies as control. There were no differences between DR-induced and DQ-induced cytokine productivity from monocytes.

In this regard, the present findings are not in agreement with those of some previous studies showing that IL-12 mRNA is more highly expressed in tuberculoid lesions as compared to lepromatous lesions (2, 3). According to the results of these previous studies, tuberculoid patients are supposed to allow *M. leprae* infection although they have the ability to produce a suf-

TABLE 1. *IL-1β and IL-12 production from monocytes by signaling via HLA-II molecules*

Clinical type	IL-1β (pg/ml)	IL-12 (pg/ml)	IFN-γ/IL-12
L-lep	322±107	402±299 †	2.93±2.89 ‡
T-lep	264±138	110±119 †	28.03±8.71 ‡
HC	418±340	304±225	N.D

The amounts of cytokines are given as the mean value of duplicate cultures with L243, after subtraction of the mean value obtained from cultures with control antibodies in each subject. L-lep, T-lep and HC represent patients with lepromatous type of leprosy, tuberculoid type of leprosy, and healthy controls, respectively.
†; Mann-Whitney *U* test, $p<0.05$.
‡; Mann-Whitney *U* test, $p<0.01$.

ficient volume of IL-12 to induce CMI responses. Moreover, *M. leprae* has the longest doubling time of all known bacteria and has massive gene decay, including the genes coding virulence determinants (8). From this viewpoint, we believe that tuberculoid patients are also susceptible to *M. leprae*. Thus, we speculate that the low productivities of IL-12 by stimulus via HLA-II molecules in tuberculoid patients might have an influence on the host defense function in early stage *M. leprae* infection.

We previously reported the lymphokine productivity of T-cell lines established from PBMCs in response to Major Membrane Protein II (MMPII), one of the outer membrane protein species derived from *M. leprae* (9). In such studies, however, it was impossible to classify leprosy patients based on IFN-γ productivity in T-cell lines. We thus considered that the balance between IL-12 from monocytes produced by stimulus via HLA class II molecules, and IFN-γ from Th cells might explain individual differences in CMI activities of clinical types of leprosy. The IFN-γ/IL-12 ratio in each subject was calculated and revealed that the ratio of lepromatous patients was significantly lower than that of tuberculoid patients ($p<0.01$). These findings indicate that insufficient IFN-γ production was induced by IL-12 in lepromatous patients, and this might reflect the abnormality of cellular immune responses observed in the lepromatous type of leprosy.

We speculate that this phenomenon might reflect the abnormality in cellular immune responses seen in the lepromatous type of leprosy. In this study, however, we did not evaluate the cytokine productivity from monocytes using antigen-specific stimuli. Further studies are needed to clarify whether the difference of IFN-γ/IL-12 ratio between lepromatous and tuberculoid patients depends upon the antigen-specific immune responses.

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HLA-mediated signaling via HLA-peptide-TCR complex determines immune responses of antigen-presenting cells

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ABSTRACT

When HLA-DR, -DQ and -DP molecules were cross-linked by solid-phase mAbs, monocytes produced monokines, and only anti-DR Ab markedly activates MAP kinase Erk, whereas anti-DR, anti-DQ and anti-DP all activate MAP kinase p38. DR-restricted T cells that are established from PBMC and are reactive with mite antigens, PPD and random 19-mer peptides, exhibited higher IFN-γ : IL-4 ratio than did DQ- or DP-restricted T cells. These results indicate that HLA-DR, -DQ and -DP molecules transmit distinct signals to monocytes via MAP kinases and lead to distinct monokine activation patterns, which may affect T-cell responses *in vivo*. Thus, the need for generation of a multigene family of class II MHC seems apparent. HLA-DR on B cells, on the other hand, not only present antigenic peptides to T cells, but also up-regulate IgM production, in association with Syk activation. When HLA-DR or CD3 molecules on cloned $CD4^+$ T cells were cross-linked by solid-phase mAbs, T cells proliferated, and this resulted in anergy. We propose that signaling via HLA-DR molecules on $CD4^+$ T cells at least in part contributes to the induction of T cell anergy that can be induced by soluble form of antigenic peptide. We next used IFN-γ-treated and irradiated periodontal ligament fibroblasts (PDL) expressing HLA-DR molecules. Indeed, Th cells did not show proliferative responses when peptide-pulsed PDL were used as APC, whereas PDL produced larger amounts of IL-6, IL-8, MCP-1 and RANTES compared with controls, when cultured with anti-HLA-DR mAb or emetine-treated T cells. These findings suggest that HLA-DR expressed on fibroblasts may act as receptor molecules that transmit signals into fibroblasts, based on DR-peptide-TCR interaction, resulting in the secretion of several cytokine species.

INTRODUCTION

We earlier reported that interactions between a $CD4^+$ T cell clone and monocyte via altered TCR ligands, affect monocyte responses to produce IL-12 with marginal involvement of CD40, events which lead to specific up-regulation of IFN-γ production from T cells (1). Thus, signals transmitted to monocytes via class II HLA molecules are involved in determining immune response patterns. It is highly conceivable that signals transmitted by class II MHC molecules in B cells, in regulating APC function during cognate T-B-cell interactions, are important, for the following reasons: (*a*) cross-linking class II molecules induces an increase in intracellular calcium and cAMP in mouse or human B cell lines (2-5); (*b*) class II MHC-mediated signals lead to homotypic aggregation of B cells (6); (*c*) cross-linking HLA-DR molecules on B cells induces apoptosis (7); (*d*) class II MHC molecules

without the intracellular domain expressed on B lymphoma cells will not lead to an increase in cAMP and subsequent CD80 up-regulation, when stimulated with a CD28-expressing autoreactive T hybridoma cells (8); (*e*) cytoplasmic domain mutants of class II MHC abrogate generation of intracellular cAMP (9) and translocation of PKC (10); and (*f*) cross-linking HLA-DR molecules expressed on B cells induces phosphorylation of Src family kinases (Lyn, Fgr) (11) and Syk (12). Moreover, engagement of class II molecules on the THP-1 monocyte cell line with Staphylococcal enterotoxin A induced IL-1β and TNF-α (13). While functional consequences of such DR-mediated signaling events induced by T cells are largely unknown, these observations do raise the possibility that signaling through class II MHC molecules may affect monocyte responses as well, including monokine secretion, upon TCR-TCR ligand interaction.

Our previous investigations on HLA-DR vs -DQ (14) or on I-A vs I-E by others (15) suggested their distinct roles in activating Th / Ts. Thus, HLA-DR function as an Ir-gene for shistosomal antigen-specific immune responses, whereas HLA-DQ do as an Is-gene, being epistatic to DR. However, their roles in activating Th1 / Th2 have remained elusive. To investigate the consequence of signaling events through distinct subregion products of class II HLA, we first tested monokine secretion patterns induced by (*a*) solid-phase mAbs to HLA-DR, -DQ and -DP molecules expressed on peripheral blood adherent monocytes, and (*b*) co-culture of peptide-pulsed monocytes with emetine-treated T cell clones of various HLA restriction patterns (16).

MATERIALS AND METHODS

Reagents

Anti-HLA class II mAb HU4 (anti-HLA-DRB1+DRB5 IgG2a, monomorphic), L243 (anti-HLA-DRB1+DRB4 IgG2a, monomorphic), HU11 (anti-HLA-DQ4+5+6 IgG2a), HU18 (anti-HLA-DQ7+8+9 IgG2a) or B7/21 (anti-HLA-DP IgG1, monomorphic) (1) were as described. Anti-HLA class II mAbs 1a3 (anti-HLA-DQ IgG2a, monomorphic) (Leinco Technologies, Inc. Manchester, UK) were purchased. Mouse IgG, IgG1, and IgG2a were purchased for control, from BioPur AG (Bubendorf, Switzerland) and Biogenesis (Poole, UK). Abs were purified from the ascites-form of mAbs, using a Protein-A column (PIERCE, IL). F(ab')2 fragments of L243 and mouse IgG were prepared, using ImmunoPure F(ab')2 Preparation Kits (Pierce) with extensive dialysis to remove residual Fc fragments. Genistein (Sigma, St. Louis, LA), GF109203X (Sigma), piceatannol (Sigma), PD98059 (New England Biolabs, Beverly, MA) and SB203580 (Calbiochem, La Jolla, CA) were purchased. *Dermatophagoides farinae* (*Der f*) antigens were kindly provided by Torii Pharmaceuticals (Tokyo, Japan). PPD was purchased from Japan BCG Laboratory (Tokyo, Japan). Peptides with defined sequences were synthesized using a solid-phase simultaneous multiple peptide synthesizer PSSM-8 (Shimadzu Corp., Kyoto, Japan) based on the Fmoc strategy and using a ten-fold molar excess of single Fmoc-amino acids, then were purified using C18 reverse-phase high-performance liquid chromatography. In the case of degenerate peptides, the introduction of randomized sequence positions was done in a double coupling step with equimolar mixtures of Fmoc-L-amino acids, used in an equimolar ratio with respect to coupling sites of the resins (all positions have 19 amino acid residues except Cys).

Human T cell clones

Human CD4+ T cell clone BC20.7 that recognizes DR14 (DRA + DRB1*1405) + residues 84-100 of BCGa protein (BCGap84-100; EEYLILSARDVLAVVSK), has been described previously (17). OT1.1 (18) and DT13.2 (1) are specific for DP5 (DPA1*0201 + DPB1*0501) + p53p153-165 (STPPPGTRVRAMAIYKQS) and DQ6 (DQA1*0102 + DQB1*0602) + *Der f* Ip18-31 (RSLRTVTPIRMQGG), respectively. T cell clones were fed weekly with 50 U/ml human rIL-2 and 10 U/ml human rIL-4, in the presence of irradiated autologous PBMC prepulsed with each peptide, in RPMI 1640

medium (Gibco, Grand Island, NY) supplemented with 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% pooled, heat-inactivated normal human male plasma in 24-well flat-bottomed culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ). Culture medium and Ab preparations tested for contamination with endotoxin, exhibited negative results. Human bleeding and animal experiments (ascites preparation) were in accordance with institutional guidelines.

Preparation of adherent APC

PBMC were freshly prepared from heparinized blood of healthy adult donors, using Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ). The PBMC were incubated at 3 x 10^7 cells in 10 ml of 10% HS/RPMI for 1.5 h in 90-mm culture-grade plastic petri dishes pre-coated with heat-inactivated autologous plasma, at 37°C in a CO2 incubator. After removing non-adherent cells, the adherent cells were recovered from plates by incubating with ice-cold 0.05% EDTA/PBS for 10 min and repeated pipeting. Monocytes were cultured for 48 h to allow adherence-induced transcription of monokine mRNA to subside (19). This population was composed principally of monocytes and were > 90% CD14-positive, as analyzed by FACS (not shown). *HLA class II* (*DR*, *DQ* and *DP*) alleles were determined, as described elsewhere (1). HLA type of the two monocyte donors were DRB1*0101/1201 and DRB1*1405/1502, both of which are negative for DRB4.

Stimulation of monocytes

Ten μg/ml anti-DR Ab (L243), anti-DQ Ab (1a3), anti-DP Ab (B7/21), mouse IgG (alternatively, IgG1 and IgG2a) were pre-coated onto 96-well flat-bottomed culture plates. Adherent cells were incubated at 6 x 10^4 cells/well where mAbs are immobilized, at 37°C in a CO2 incubator. Culture supernatants were collected at 6, 16, 24, 48 and 72 h and stored in aliquots at -80°C until determinations of lymphokine concentrations.

Alternatively, T cells treated with 0, 10, 30 and 90 μg/ml of *de novo* protein synthesis inhibitor emetine (Sigma) (20) for 1 h at 37°C were washed three times with RPMI 1640 medium. Cells were re-suspended in culture medium, incubated for 3 h at 37°C, then washed three times with RPMI 1640 medium and co-cultured with peptide-pulsed or mock-pulsed monocytes. Culture supernatants after 16- (for IL-12), 24- (for IL-1β, IL-10, IL-18, GM-CSF and TNF-α) and 48-h (for IL-6) incubation were collected, and subjected to ELISA. Treatment of T cells by emetine abrogated IL-4 production from BC20.7 (BCGa-specific, DR14-restricted), in a dose-dependent manner; ninety μg/ml of emetine treatment resulted in a complete abrogation of IL-4 production, but not IL-12 produced by peptide-pulsed monocytes (not shown). Moreover, culture supernatants of the peptide-pulsed monocytes stimulated with emetine-treated T cells were positive for IL-12 production, but not so mock-pulsed monocytes stimulated with emetine-treated T cells (not shown). The interaction between HLA and peptide alone did not induce monokine production. Results were similar in case of HLA-DQ-restricted DT13.2 and HLA-DP-restricted OT1.1 (not shown).

ELISA assays

The human IL-4, IFN-γ, IL-1β, 10, 12 (p40 + p70), GM-CSF and TNF-α ELISA kits (Biosource International) and human IL-6 ELISA kit (Genzyme) were used for quantitation of lymphokines in the supernatants, according to manufacturer's instructions. ELISA kit for IL-18 was kindly provided by Dr. M. Kurimoto (Hayashibara Biochemical Laboratories, inc., Okayama, Japan). Statistical significance was analyzed using Student's t test.

Western immunoblot analysis

Monocytes prepared from PBMC were added to 96-well culture plates in which class II HLA mAbs had been immobilized, followed by centrifugation. After 10-60-min incubation at 37°C, ice-cold 100 μM sodium vanadate/PBS was added for washing, followed by lysing in 50 μl of lysing buffer (150 mM NaCl, 20 mM Tris, pH7.6, 0.5% Nonidet P-40, 2 mM Na-orthovanadate, 1 mM NaF, 5 mM EDTA plus a protease inhibitor cocktail purchased from

SIGMA). After centrifugation, supernatant fluids of the lysates were electrophoresed on SDS-PAGE gels and transferred to nitrocellulose membrane. After blocking with 10% skim milk, 0.2% Tween-20 in Tris-buffered saline, the membrane was incubated with Abs specific for Erk, JNK and p38 (Santa Cruz) or with Abs specific for the activated form of Erk, JNK and p38 (Upstate Biotechnology, NY), washed extensively and subjected to chemiluminescence detection with peroxidase-conjugated anti-mouse IgG Ab, using an ECL kit (Amersham, U.K.). Signals were analyzed using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov.).

Establishment and analysis of Der f-, PPD- and X19-reactive T cell lines

Derf (crude mite antigen)-specific short-term T cell lines were established from PBMC from two donors carrying different HLA types (MA: HLA-DRB1*1405 / DRB1*1502, NI: HLA-DRB1*0901 / DRB1*1302). HLA-DR-restricted and HLA-DP-restricted T cell lines were established by co-culture either with anti-HLA-DQ (HU11 and/or HU18) + anti-HLA-DP (B7/21) mAbs or with anti-HLA-DR (HU4 and L243) + anti-HLA-DQ (HU11 and/or HU18) mAbs, respectively, in the presence of the crude extract of *Derf*. Restriction molecules of these cell lines were confirmed by inhibition assays with mAbs (not shown), and all the cell lines of expected restriction patterns were used for the analysis. These cell lines were restimulated with excess concentrations of antigens (10 μg/ml), then after 48-h incubation, culture supernatants were collected for measurements of IFN-γ and IL-4 production by ELISA. PPD-specific short-term T cell lines were established from PBMC of donor MA. HLA-DR-restricted and HLA-DQ-restricted T cell lines were established by co-culture with anti-HLA-DQ (HU11) + anti-HLA-DP (B7/21) mAbs or anti-HLA-DR (HU4 and L243) + anti-HLA-DP (B7/21) mAbs, respectively, in the presence of PPD. X19 (19-mer peptides with random sequences)-reactive T cell clones were established from PBMC of donor MA, using X19, IL-4, IL-7, IL-9, IL-15 and agonistic Ab to CD29, under cloned. Restriction molecules were determined by inhibition assays with mAbs.

In vitro immune-complex kinase assay

The human B lymphoblastoid cell line LD2B (1×10^7) was incubated for 10 min on ice and then pre-incubated either with biotinylated Igs (40 μg / 200 μl) or with biotinylated F(ab')2 fragments (12 μg / 200 μl) for 10 min on ice. After washing with ice-cold RPMI1640, the cells were suspended with 50 μl of 10 % FCS / RPMI and cross-linked with 50 μl of avidin (1 mg / ml). After 10-min incubation at 37°C, ice-cold 100 μM Na_3VO_4 / PBS was added, followed by pelleting and lysing in 400 μl of the lysing buffer. Supernatant fluids of the lysates were pre-cleared with Protein A-agarose beads, then were incubated with a rabbit polyclonal anti-Syk Ab (Santa Cruz Biotechnology, Inc.), using Protein A-agarose beads (PIERCE). After shaking for 30-min at 4°C, the beads were washed 4 times with lysis buffer. An aliquot of immunoprecipitated proteins was eluted with Laemmli buffer containing 2-ME, for immunoblotting analysis. Residual beads were washed once with kinase buffer (25 mM HEPES [pH7.4], 0.1%[v/v] Nonidet P-40, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 30 μM Na_3VO_4; 30) and were re-suspended in 30 μl of the kinase buffer containing 2 μg (0.11 nmol) MBP (SIGMA), in the presence of either 27.5 nmol HS1 peptide or an irrelevant peptide. Reactions were initiated by adding 3.75 μM [γ-^{32}P]ATP (10 μCi of [γ-^{32}P]ATP/sample, 5000 Ci/mmol; Amersham), then incubated for 2.5 min at 25 °C. The reactions were terminated by adding an equal volume of 2x Laemmli buffer. The supernatants were boiled for 2 min and applied to a 12% SDS-PAGE. After electrophoresis, the gel was fixed and vacuum dried, and analyzed using a bio-imaging analyzer (BAS2000, Fuji Film, Tokyo). Eluted protein samples were separated on 7.5 % SDS-PAGE and transferred to nitrocellulose membrane. After blocking with 10 % skim milk, 0.2 %