used were anti-MAGE-A1 (clone MA454), anti-MAGE-A3 (clone M3H67), anti-MAGE-A4 (clone 57B), anti-CT7/MAGEC1 (clone CT7-33) and anti-CT10/MAGEC2 (clone LX-CT10.5). For cancer-testis (CT) antigens, only strong nuclear and/or cytoplasmic staining as observed in testicular tissue (positive control) in at least 5% of cells was scored as positive. 57B and M3H67 mAbs generated against MAGE-A3 and MAGE-A4 recombinant proteins, respectively, were both shown to recognize multiple MAGE-A family molecules.[35,36]

Table 1. Heteroclitic antibody response and clinical response after CHP-NY-ESO-1 vaccination

ID	Heteroclitic response No. of antigens	Weeks (the No.)	Clinical response
E-1	0	89 (31)	Regression
E-2	2	14 (7)	Partial regression
E-3	1	28 (12)	Stable
E-4	3	12 (6)	Progressive
E-5	2	22 (11)	Partial regression
E-6	0	4 (3)	N.E.
E-7	4	2 (2)	N.E.
E-8	7	54 (27)	Stable
P-2	1	28 (10)	PSA stabilization
P-3	2	29 (13)	PSA stabilization

Abbreviations: Weeks (the No.): weeks after the start of vaccination and the number of vaccinations given; N.E.: not evaluable.

Reverse transcription-polymerase chain reaction

Total cellular RNA was extracted from frozen tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA). Conventional reverse transcription-polymerase chain reaction (RT-PCR) was performed against NY-ESO-1, LAGE-1, MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1, SOX2, SSX2 and XAGE1B.[30,31]

Results

Antibody response against 13 tumor antigens in CHP-NY-ESO-1-vaccinated patients

We analyzed antibody responses against NY-ESO-1, NY-ESO-1-related antigen LAGE-1, other CT antigens MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1, SSX2 and XAGE1B, SOX2 and p53 in esophageal cancer patients E-1, E-2, E-3, E-4, E-5, E-6, E-7 and E-8 and prostate cancer patients P-2 and P-3 before and after a cycle of CHP-NY-ESO-1 vaccination (Fig. 1 and Table 1). Before vaccination, strong antibody responses against NY-ESO-1 and/or LAGE-1 were observed in E-2 and P-3 and defined as baseline seropositive. Additionally,

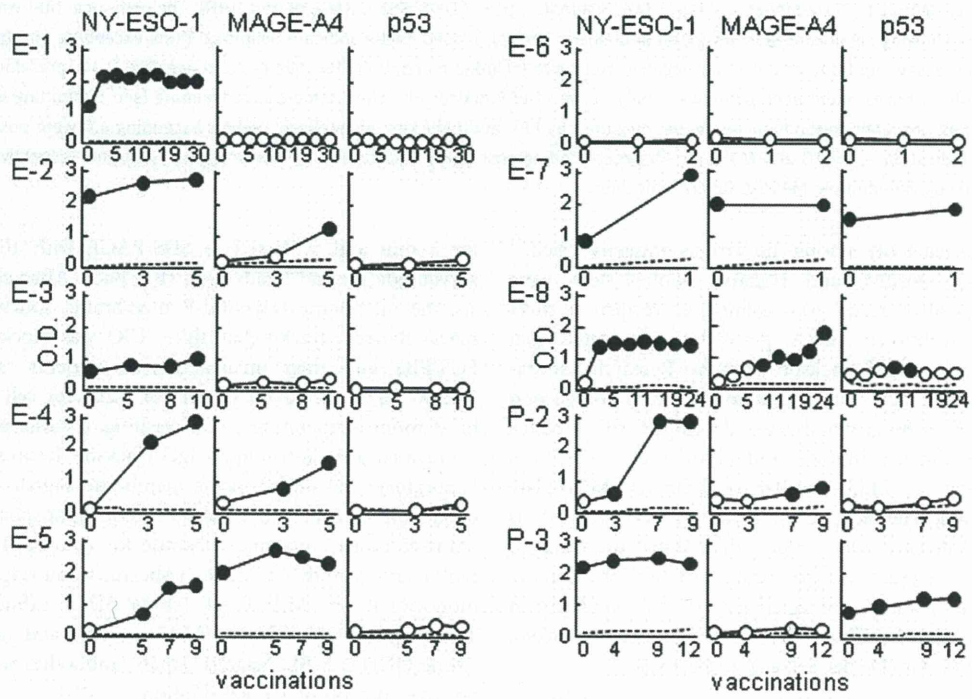

Figure 2. IgG antibody response against NY-ESO-1, MAGE-A4 and p53 in sera from patients before and after CHP-NY-ESO-1 vaccination by ELISA. Sera diluted at 1:100 were assayed against N-His6-tagged recombinant proteins NY-ESO-1, MAGE-A4 and Akt produced in *E. coli* and recombinant proteins p53 and CCDC-62 produced in *Baculovirus*. Akt and CCDC-62 were included as negative control (dotted line). Positive reaction (closed circles) represented the OD values exceeding three times the control OD value.

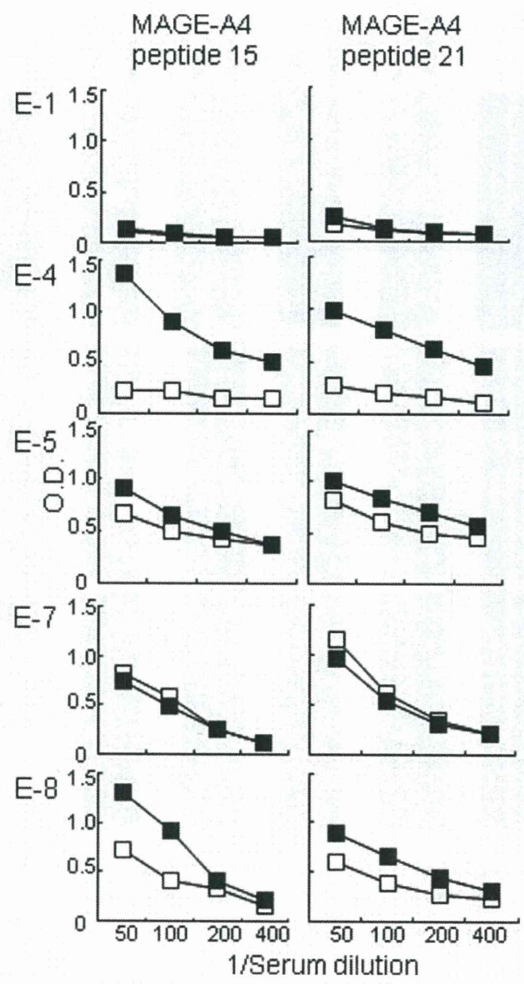

Figure 3. Serially diluted sera from patients before (open squares) and after (closed squares) CHP-NY-ESO-1 vaccination were assayed against MAGE-A4 peptide 15 and peptide 21 by IgG ELISA.

marginal antibody responses were observed in E-1, E-3 and E-7. In E-5, antibody against MAGE-A1, MAGE-A3, MAGE-A4 and SOX2 was observed. In E-7, antibody against MAGE-A4, CT7/MAGEC1, p53 and SOX2 was observed. In P-3, antibody against p53 was observed. After vaccination, in all patients except E-6, antibody response against NY-ESO-1 and LAGE-1 was increased or induced. In E-2, antibody responses against MAGE-A3 and MAGE-A4 were induced. In E-3, antibody response against SOX2 was induced. In E-4, antibody responses against MAGE-A3, MAGE-A4 and CT10/MAGEC2 were induced. In E-5, antibody responses against MAGE-A3 and MAGE-A4 were increased. In E-7, antibody responses against CT7/MAGEC1, p53 and SOX2 were increased and that against CT10/MAGEC2 was induced. In E-8, antibody responses against MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT45, CT46/HORMAD1 and p53 were induced. In P-2, antibody response against MAGE-A4 was induced. In P-3, antibody response against CT7/MAGEC1 was induced and that against p53 was increased. No antibody against DHFR included as a control was detected in any patient. Furthermore, no increase of antibody response was observed against EBV and CMV after CHP-NY-ESO-1 vaccination.

Expression of 13 tumor antigens in tumor specimens

Expression of NY-ESO-1 was detected by RT-PCR and IHC in tumors from all patients before vaccination. Expression of other tumor antigens except p53 was analyzed by RT-PCR in E-1, E-5, E-6, E-7 and E-8, and expression of MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1 and CT10/MAGEC2 was also analyzed by IHC in E-1, E-2, E-4, E-5, E-6, E-7 and E-8 (Fig. 1 and Supporting Information Table). Mutation of p53 was not determined in our study. Expression of corresponding antigen was confirmed with tumor specimens in patients who showed antibody against tumor antigens.

Antibody response against tumor antigens in CHP-NY-ESO-1-vaccinated patients: No involvement of antibody against His6-tag and the product of E. coli present in the vaccine

Antibody responses against selected tumor antigens were further confirmed in sera obtained at each time during multiple vaccinations. As shown in Figure 2, IgG antibody against MAGE-A4 was detected in sera from E-5 and E-7 before vaccination, and the response was increased or induced in E-2, E-4, E-5, E-8 and P-2 after vaccination. IgG antibody against p53 was detected in sera from E-7 and P-3 before vaccination, and the response was increased or induced in E-7, E-8 and P-3.

Induction of IgM antibody against MAGE-A4 was detected in sera from E-8 after vaccination (Supporting Information Fig. 1). IgM antibody against p53 was detected in sera from E-7 before vaccination. Increase or induction of IgM antibody against p53 was detected in E-7 and E-8 after vaccination. Interestingly, in E-8, transient IgM response against MAGE-A4 and p53 was followed by IgG response.

Recombinant NY-ESO-1 protein used for vaccination has His6-tag in the N-terminus and was produced in *E. coli* as the host cells. All antigens shown in Figure 1 also have His6-tag and were produced in *E. coli*. To exclude the possibility of detecting antibody against His6-tag and/or the product of *E. coli* in the assay that might be raised by vaccination, DHFR was tested as control. No antibody against DHFR was detected (see above). To further exclude the possibility, the antibody response against control antigens was examined by IgG ELISA using serum samples obtained in each time during multiple vaccinations. As shown in Figure 2, antibody against Akt protein with His6-tag and produced in *E. coli* was within a background level (<0.2 OD value). p53 used in the experiments shown in Figure 2 and Supporting Information Figure 1, but not in Figure 1, and CCDC-62 protein

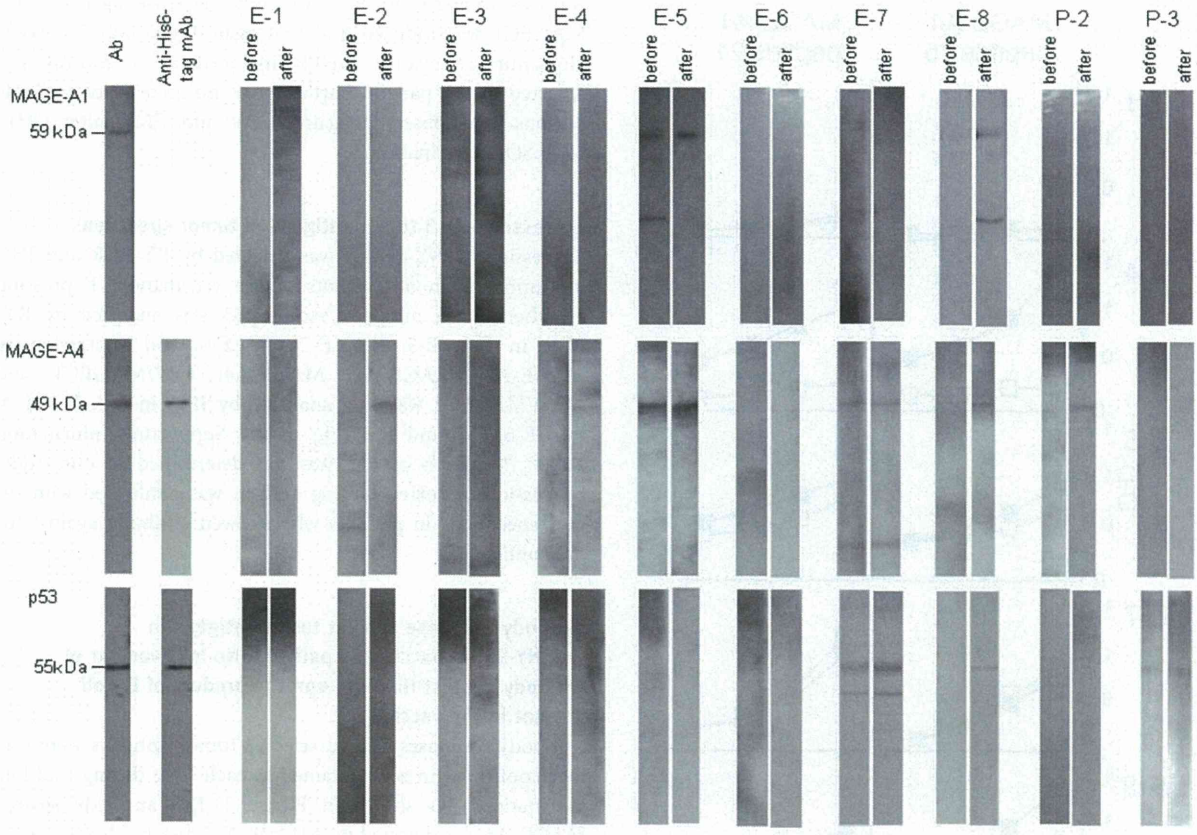

Figure 4. Western blot analysis. Reaction of sera against MAGE-A1, MAGE-A4 and p53 was investigated. Recombinant proteins (20 ng) were run by SDS-PAGE and transferred to a membrane by electrophoresis. Sera (1:1,000) from all patients obtained before and after vaccination were examined. Marker and control bands of each protein detected by monoclonal or polyclonal antibody (1:1,000) are also shown.

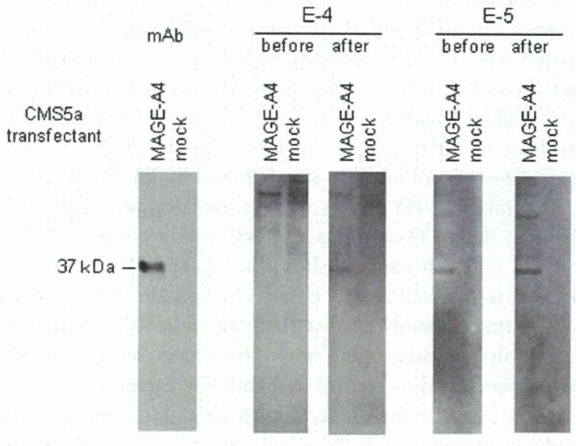

Figure 5. Western blot analysis of sera against MAGE-A4 in lysate of MAGE-A4-transfected CMS5a cells. Cell lysate (20 μg) was run by SDS-PAGE, transferred to a membrane by electrophoresis and sera (1:200) from E-4 and E-5 patients obtained before and after CHP-NY-ESO-1 vaccination were examined. Control band of the protein detected by monoclonal antibody (1:1,000) is shown.

share His6-tag and were produced by *Baculovirus*. Antibody against CCDC-62 was undetectable in sera from any patients.

Next, we synthesized MAGE-A4 OLPs and investigated antibody response by ELISA. Antibody response against MAGE-A4 peptides 15 and 21 was frequently observed in patients showing antibody response against MAGE-A4 protein (Supporting Information Fig. 2). Serially diluted sera from patients E-4, E-5, E-7 and E-8 obtained before and after CHP-NY-ESO-1 vaccination were examined against MAGE-A4 peptides 15 and 21 by IgG ELISA (Fig. 3). Increase or induction of antibody response was observed in E-4, E-5 and E-8, but not E-7 after vaccination. No antibody response was detected in E-1 included as negative control. These results were consistent with those by ELISA using recombinant MAGE-A4 protein in Figure 2.

Western blot analysis

The specificity of antibody against MAGE-A1, MAGE-A4 and p53 in sera from all patients vaccinated was further analyzed by Western blot (Fig. 4). Each antibody as positive control showed the representative band for MAGE-A1 protein at 59 kDa, for MAGE-A4 protein at 49 kDa and for p53 protein

at 55 kDa. Increase of reaction with the bands was observed with recombinant MAGE-A1 protein in sera from E-8, with recombinant MAGE-A4 protein in sera from E-2, E-4, E-5, E-8 and P-2 and with p53 in sera from E-7, E-8 and P-3 obtained after vaccination.

Specificity of the reaction was further confirmed using transfectants. As shown in Figure 5, sera from E-4 after vaccination and from E-5 before and after vaccination reacted to MAGE-A4 in lysate of MAGE-A4-transfected murine fibrosarcoma CMS5a cells. No reaction was observed with lysate of mock-transfected CMS5a cells.

Discussion

Efficient elicitation of host immune response is a prerequisite for successful immunotherapy using cancer vaccine, and immune monitoring of specific antibody, CD4 and CD8 T cell responses against tumor antigens after vaccination is crucial to evaluate the response. In our study, we investigated antibody response against 13 tumor antigens by ELISA using recombinant proteins to evaluate the immune response more precisely. Nine of ten patients analyzed except E-6 showed an increase or induction of antibody response against NY-ESO-1 and its related LAGE-1 antigen after CHP-NY-ESO-1 vaccination. Eight of these nine patients showed an increase or induction of antibody response to either of these antigens after vaccination. Previously, it was reported that sera from patients vaccinated with recombinant NY-ESO-1 protein and CpG in Montanide sometimes showed nonspecific production of antibody against other recombinant proteins used for control,[11,37] and some of these responses could be attributed to reactivity against bacterial components or His6-tag. To address this possibility, we performed specificity analysis of the antibody response using control recombinant proteins, synthetic peptides and by Western blot that showed heteroclitic responses were not against His6-tag and/or bacterial products included in a preparation of CHP-NY-ESO-1 used for vaccination.

We reported previously that those patients showed NY-ESO-1 specific antibody and CD4 and CD8 T cell responses during vaccination.[14,15] The findings suggest that increase or induction of antibody response against tumor antigens, *e.g.*, MAGE-A3 and MAGE-A4, as well as NY-ESO-1 after CHP-NY-ESO-1 vaccination may be caused by their release from tumor cells damaged by NY-ESO-1-specific immunity. Therefore, antibody response to multiple tumor antigens may suggest an intensity of the overall host immune response against the tumor, and detection of multiple heteroclitic serological responses using a panel of recombinant proteins would be a new tool of immunological monitoring for antitumor responses. A clear correlation between heteroclitic antibody responses and clinical outcomes could not be established in the limited number of patients analyzed in our study (Table 1). However, antibody response as well as CD4 and/or CD8 T cell responses to heteroclitic tumor antigens would be useful for evaluating overall immune response to tumor.

A number of studies have shown the relationship between heteroclitic immune response and clinical response. Germeau *et al.*[19] reported that the frequency of CTL precursor increased tenfold in some patients after vaccination using MAGE antigenic peptides, although they found no significant difference in the levels against immunizing antigens between the tumor-regressor and -progressor patients. They then analyzed CTL precursors against other tumor antigens than that utilized for vaccine and found that the immune responses elicited to those irrelevant antigens after vaccination might contribute to the whole immune response to a given tumor and was correlated to clinical responses. Similarly, Butterfield *et al.*[23,24] reported that peptide-specific T cell response was efficiently induced in most patients by immunization with MART-1/Melan-A peptide pulsed dendritic cells. However, cellular immune responses against not only MART-1/Melan-A but also gp100 and tyrosinase were detected only in a complete clinical responder. These findings suggest a relationship between heteroclitic CTL responses and clinical responses. Furthermore, Disis *et al.* reported induction of both cellular and humoral responses against other intramolecular determinants in patients immunized with HER-2/neu peptide vaccine, and of antibody response to p53 in patients immunized with HER-2/neu peptide vaccine.[17,22] They further studied the effect of HER-2/neu T-helper peptide-based vaccinated patients receiving trastuzumab therapy and observed prolonged immune responses against not only the vaccine antigen but also cryptic antigens.[38] Collectively, the presence of either humoral or cellular immune response to multiple tumor antigens appears to be indicative of the strength of overall response against the tumor and predictive of clinical response. In our study, we used a panel of 13 tumor antigens for the detection of the humoral response. Serological detection of responses to multiple tumor antigens that were shown to be highly immunogenic in cancer patients would be convenient and could be included in routine immune monitoring.

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Analysis of VH Gene Rearrangement and Somatic Hypermutation in Sjogren's Syndrome and IgG4-Related Sclerosing Sialadenitis

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Abstract

IgG4-related sclerosing sialadenitis is currently considered as an autoimmune disease distinct from Sjogren's syndrome (SS) and responds extremely well to steroid therapy. To further elucidate the characteristics of IgG4-related sclerosing sialadenitis, we analysed VH fragments of IgH genes and their somatic hypermutation in SS (n = 3) and IgG4-related sclerosing sialadenitis (n = 3), using sialolithiasis (n = 3) as a non-autoimmune control. DNA was extracted from the affected inflammatory lesions. After PCR amplification of rearranged IgH genes, at least 50 clones per case (more than 500 clones in total) were sequenced for VH fragments. Monoclonal IgH rearrangement was not detected in any cases examined. When compared with sialolithiasis, there was no VH family or VH fragment specific to SS or IgG4-related sclerosing sialadenitis. However, rates of unmutated VH fragments in SS (30%) and IgG4-related sclerosing sialadenitis (39%) were higher than that in sialolithiasis (14%) with statistical significance (P = 0.0005 and P < 0.0001, respectively). This finding suggests that some autoantibodies encoded by germline or less mutated VH genes may fail to be eliminated and could play a role in the development of SS and IgG4-related sclerosing sialadenitis.

Introduction

Chronic sclerosing sialadenitis, also known as a Kuttner tumour, is a benign inflammatory process which is usually unilateral and which occurs almost exclusively in the submandibular gland [1, 2]. It is characterized histologically by periductal fibrosis, dense lymphocytic infiltration, loss of the acini and marked sclerosis of the salivary gland. As chronic sclerosing sialadenitis manifests as a hard mass, it usually raises a strong clinical suspicion of a malignant neoplasm. Recent studies have shown that IgG4 concentrations in serum are elevated and that plasmacytic cells infiltrating the salivary glands are positive for IgG4 in chronic sclerosing sialadenitis but not in Sjogren's syndrome [3, 4], suggesting that the former involves inflammatory processes distinct from those of the latter. A dense IgG4-positive plasma cell infiltration has also been found in Mikulicz's disease, chronic sclerosing pancreatitis (or autoimmune pancreatitis) [5], IgG4-related sclerosing cholangitis [6] and other sclerosing lesions. Steroids are very effective in treating these IgG4-related disorders, and autoimmune mechanisms may play a role in their development [7].

Analysis of the immunoglobulin heavy chain gene is helpful in clarifying the characteristics of B cells infiltrating inflammatory autoimmune lesions. In this study, we analysed immunoglobulin heavy chain gene rearrangement and somatic hypermutation of SS and IgG4-related sclerosing sialadenitis, using sialolithiasis as a control.

Materials and methods

Case selection. Typical cases of primary SS (n = 3), IgG4-related sclerosing sialadenitis (n = 3) and sialolithiasis (n = 3) were recruited. None of these cases showed evidence of virus-associated hepatitis or tuberculosis. Clinicopathological data were obtained from the medical records, and the study was approved by the institutional

review board of Nagoya City University. For SS cases, biopsy specimens of the minor salivary gland of the lower lip were obtained to histologically confirm the diagnosis (focus scores for three SS cases were 4, 4 and 5, respectively) [8], and small germinal centres were present in all cases), which was further supported by the increased levels of serum anti-SS-A/Ro antibody, anti-SS-B/La antibody and rheumatoid factor. The diagnosis of SS was made according to revised Japanese criteria for SS [9]. The lip biopsy specimens were used for this study. Patients with sclerosing sialadenitis presented with painless swelling of the submandibular glands. Cryptogenic tumours were suspected, and the patients underwent surgical resection of the submandibular glands, which were subjected to examination in this study. Typical cases of sialolithiasis of the submandibular glands were resected and used as a control.

Immunohistochemical techniques. The sections were immunostained for IgG (Eu-N1; Dako, Tokyo, Japan) and IgG4 (MCO11, Binding-Site, Birmingham, UK). Infiltration of IgG-positive or IgG4-positive plasma cells was evaluated by counting the number of positive cells in ten high-power fields (×400), and the percentage of the IgG4-positive cells/IgG-positive cells was calculated in each case. Percentages of memory B and plasma cells to total B and plasma cells were calculated using immunohistochemical techniques in each case. CD27-positive B cells have been considered as memory B cells, and CD27 is positive for T, B and plasma cells [10]. We detected memory B and plasma cells using subtractive double immunostain for CD27 (137B4; Leica Biosystems Newcastle, Newcastle Upon Tyne, UK) and CD3e (SP7; Dako) as described by Steiniger *et al.* [11] with some modifications. In brief, CD27 signals were visualized first with brown chromogen using Bond Polymer Refine Detection kit (Leica Biosystems), and then, using the same tissue slides, T cells were stained using anti-CD3e antibody with purple chromogen using Bajoran Purple Chromogen System (Biocare Medical, Concord, CA, USA). Thus, only CD27-positive B and plasma cells were left to be revealed in brown colour. Total B and plasma cells were detected in serial sections using conventional immunostain for CD79a (JCB117; Leica Biosystems) [12]. After examining ten high-power fields in each case, the percentage of the memory and plasma cells to total B and plasma cells was estimated.

DNA extraction, IgH gene amplification and subcloning. Genomic DNA was extracted from formalin-fixed, paraffin-embedded sections by overnight digestion with proteinase K. DNA of all cases was found to be of satisfactory quality as confirmed by PCR for the beta-globin gene. A seminested strategy was used for PCR amplification of the VH genes using a consensus primer for conserved framework-2 (FR2A) and a consensus primer for the J region (LJH and VLJH). These primers have been used most commonly for VH gene analysis of formalin-fixed, paraffin-embedded tissue specimens tissue [13–15]. The PCR products were stained with ethidium bromide and run on agarose gels. To minimize any amplification bias, genomic DNA from each case was amplified in multiple PCR runs (n > 80), and the amplified products were mixed in one tube and then subcloned for DNA sequencing. Subcloning of the PCR products was performed with pGEM T-easy vector (Promega, Madison, WI) using DNA that was excised from a polyclonal band in the agarose gel and purified. Recombinant clones were randomly picked-up and amplified by PCR using primers encompassing the insert. Those showing the expected insert size were then sequenced using an ABI Prism Big Dye Terminator kit (Applied Biosystems, Foster City, CA) on an automatic DNA sequencer. More than 50 polyclonal clones from each case of SS, MD and chronic sialolithiasis were sequenced.

Sequence analysis. The DNA sequences were aligned with IgH sequences from IgBLAST (available at http://www.ncbi.nlm.nih.gov/igblast/). Clones that showed non-productive rearrangements were excluded from the present analysis. VH gene sequences deviating more than 2% from that of the corresponding germline gene were defined as mutated [16].

Statistical analysis. Statistical evaluation of data from the two groups was performed using Fischer's exact test (two-tailed). Analysis was performed using the statistical package JMP (SAS Institute Inc., Cary, NC, USA).

Results

Clinical data and immunohistochemistry

Clinical features of SS (n = 3), IgG4-related sclerosing sialadenitis (n = 3) and sialolithiasis cases (n = 3) are shown in Table 1. The IgG4-related sclerosing sialadenitis cases showed no xerostomia or xerophthalmia, and serum SS-A and SS-B were normal. One case involved bilateral submandibular glands (case #1), while in the other two, only one gland was affected. SS and sialolithiasis cases were typical in their clinical presentation and their histopathology. As shown in Table 1 and Fig. 1, the percentage ratio of IgG4/IgG-positive plasma cells in IgG4-related sclerosing sialadenitis tissues was more than 70%, whereas in SS and sialolithiasis, it was less than 10%.

Memory and plasma cells, as detected by subtractive double immunostains (Fig. 2), were found mainly in the areas where atrophic mucous acini and ductules were present and occasionally found in the areas where lymphoid follicles were formed. The former areas predominated over the latter in all the tissue samples studied. The percentages of memory and plasma cells to total B and plasma cells were similar in three inflammatory lesions and were

Table 1 Clinical data on Sjogren's syndrome, IgG4-related sclerosing sialadenitis, and sialolithiasis.

Case	Diagnosis	Age/sex	Xerostomia	Xerophthalmia	Serum SS-A/B	Samples	IgG4/IgG (%)	Follow-up (months)
1	SS	67/F	+	−	+/+	Lower lip	0	W/A (5)
2	SS	57/F	+	+	+/+	Lower lip	3	W/A (49)
3	SS	53/F	+	−	+/+	Lower lip	0	W/A (13)
4	IgG4 SS	65/M	−	−	−/−	Submandibular gland	84	W/A (25)
5	IgG4 SS	62/M	−	−	−/−	Submandibular gland	76	W/A (67)
6	IgG4 SS	54/M	−	−	−/−	Submandibular gland	89	W/A (49)
7	SL	73/M	−	−	−/−	Submandibular gland	6	W/A (6)
8	SL	46/F	−	−	−/−	Submandibular gland	0	W/A (15)
9	SL	48/F	−	−	−/−	Submandibular gland	8	W/A (13)

SS, Sjogren's syndrome; IgG4 SS, IgG4-related sclerosing sialadenitis; SL, sialolithiasis; W/A, alive and well.

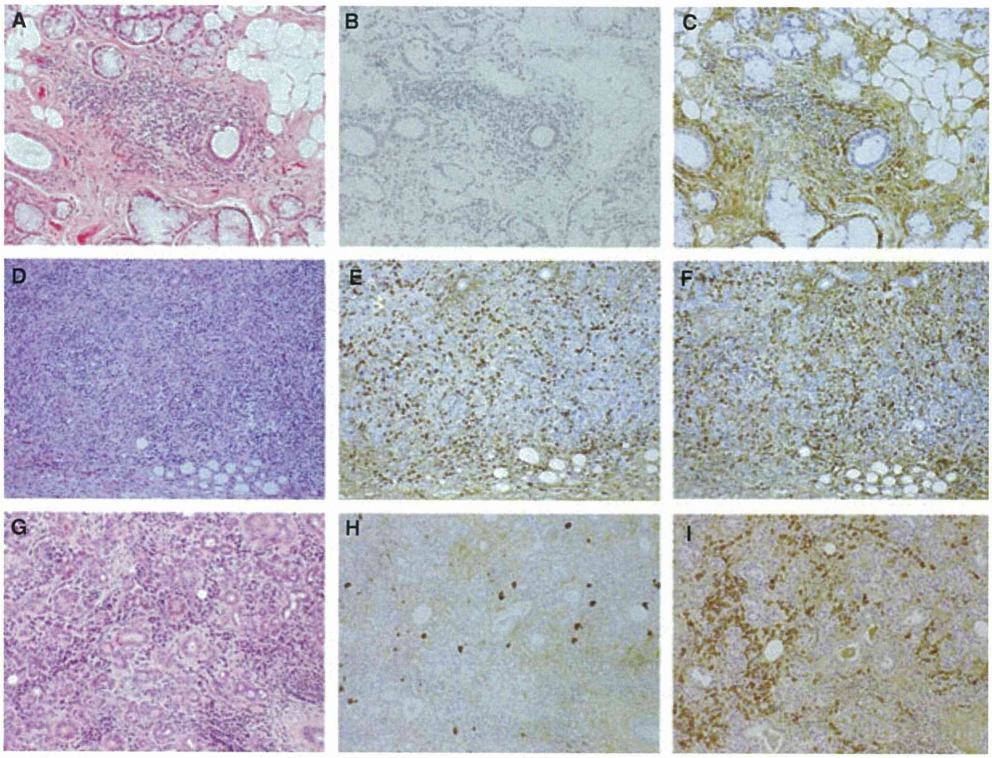

Figure 1 A–I: Histopathological findings of Sjogren's syndrome (A–C), IgG4-related sclerosing sialadenitis (D–F) and sialolithiasis (G–I). H&E stain (A, D and G); immunohistochemistry for IgG4 (B, E and H, haematoxylin counterstain) and immunohistochemistry for IgG (C, F and I, haematoxylin counterstain). Note that majority of IgG-positive plasma cells are positive for IgG4 in IgG4-related sclerosing sialadenitis.

45%, 43% and 42% for SS, IgG4-related sclerosing sialoadenitis and sialolithiasis, respectively.

VH gene fragment analysis

Monoclonal IgH rearrangement was not detected in any cases of SS, IgG4-related sclerosing sialadenitis and sialolithiasis. Sequence analyses of VH fragments are shown in Supplementary data S1–3. In SS cases, a total of 161 VH clones were sequenced for VH fragments. Among the seven VH families, the VH3 family was most frequently used in all three cases, with a rate of VH3/total clones of 64–78% (mean 72%). The VH3 family was followed in usage by the VH4 or VH1 family. Among VH3 family members, VH3-23 was the fragment most frequently used. VH clones were frequently unmutated: rates of unmutated clones relative to total clones, to VH3 family clones and to non-VH3 family clones were 30% (range, 29–31%), 36% (range, 32–43%) and 16% (range, 10–20%), respectively.

In IgG4-related sclerosing sialadenitis cases, a total of 221 clones were sequenced for VH fragments. As with SS, the VH3 family was most frequently used in all three cases of this disease, with a rate of VH3/total clones of

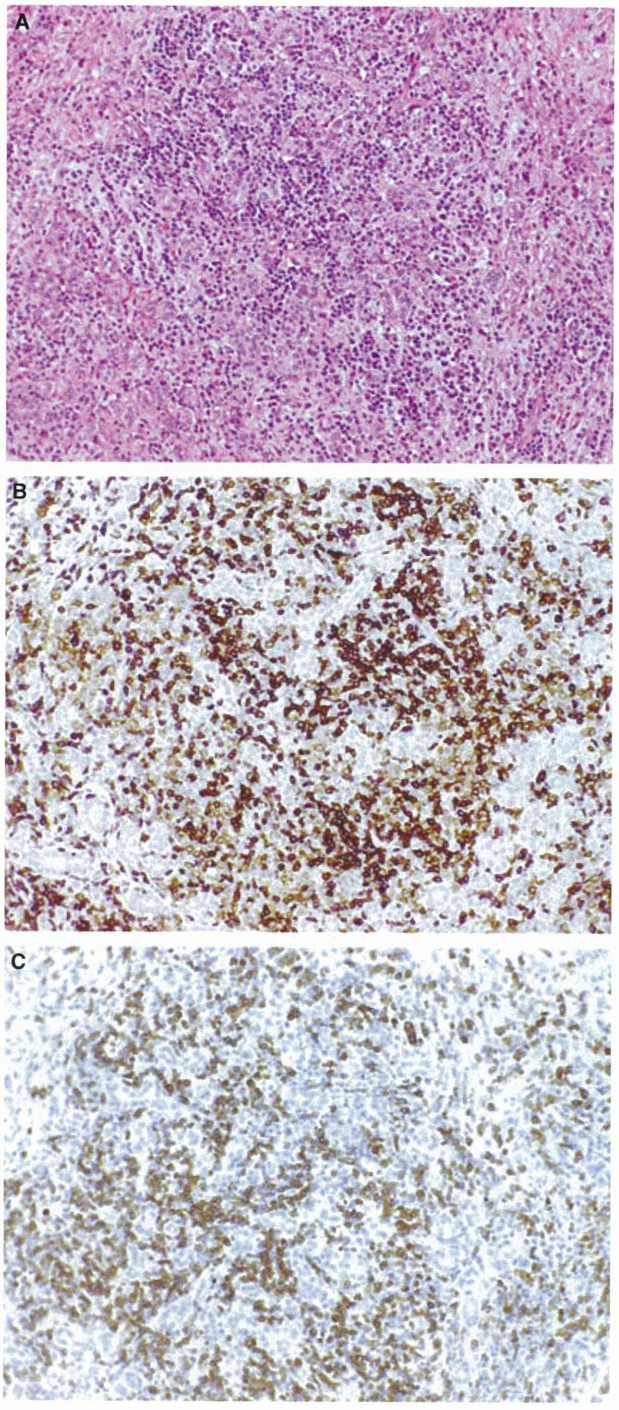

Figure 2 A–C: IgG4-related sclerosing sialadenitis. H&E (A); subtractive double immunostain for CD27 (brown) and CD3e (purple) (B, haematoxylin counterstain); and conventional immunostain for CD79a (C, haematoxylin counterstain). In subtractive double immunostains, CD27-positive T cells are masked by CD3e purple chromogen, and CD27-positive B and plasma cells are left to be revealed in brown colour.

70–76% (mean 72%). The VH3 family was followed in usage by the VH4 or VH1 family. Among VH3 family members, VH3-23 consistently emerged as the most frequently used fragment. The VH fragments were often unmutated: the rates of unmutated clones relative to total clones, to VH3 family clones and to in non-VH3 family clones were 39% (range, 37–42%), 47% (range 42–50%) and 16% (range, 10–24%), respectively.

Among the sialolithiasis cases, VH3 family clones were consistently the most frequent (mean 75%, range 74–75%), and VH3-21 and VH3-23, VH3-23 and VH3-30 were selected the most frequently in sialolithiasis case #1, #2 and #3, respectively. In addition, the rates of unmutated clones were low in this disorder when compared with SS and IgG4-related sclerosing sialadenitis: the rates of unmutated clones relative to total clones, to VH3 family clones and to non-VH3 family clones were 14% (range, 12–15%), 15% (range, 14–15%) and 11% (0–18%), respectively.

A statistical comparison is presented in Table 2. When compared with sialolithiasis (non-autoimmune control), VH clones of SS were frequently unmutated ($P = 0.0005$) as they were with IgG4-related sclerosing sialadenitis ($P < 0.0001$). For VH3 family clones, rates of unmutated clones in cases of SS and IgG4-related sclerosing sialadenitis were significantly higher than in the sialolithiasis cases ($P = 0.002$ and $P < 0.0001$, respectively). In contrast, there were no significant differences in non-VH3 family clones.

Discussion

In our study, we retrieved typical clinical cases of SS, IgG4-related sclerosing sialadenitis and sialolithiasis. We then analysed VH fragments of B cells infiltrating these three types of lesions. After PCR amplification of rearranged IgH genes, at least 50 clones per case and more than 500 clones in total were sequenced for VH fragments, and the data obtained showed that VH fragments of SS and IgG4-related sclerosing sialadenitis cases were frequently unmutated. We employed sialolithiasis tissues as a non-autoimmune control and observed chronic inflammation together with many mature lymphoid and plasma cells. In previous VH analyses [17, 18], peripheral blood B cells have been used as a control. However, as about 70% of peripheral blood B cells are naïve or unmutated [19], we consider that local non-specific inflammatory lesions (e.g. those of sialolithiasis) would be a more appropriate control in analysing local inflammation in autoimmune diseases.

Hansen *et al.* reported that the VH3 family was preferentially used in a patient with SS (VH3 > VH1 ≥ VH4 > others) [18]. In this study, a similar VH usage was observed in SS and IgG4-related sclerosing sialadenitis cases: the VH3 family was the most frequently used and VH3-23 was the most often used among VH3 fragments. However, this usage of the VH3 family and a tendency towards use of VH3-23 was also found in the sialolithiasis controls, suggesting that the

Table 2 Unmutated clone ratios in Sjogren's syndrome, chronic sclerosing sialadenitis, and sialolithoasis.

	Total			VH3 family			Non-VH3 family		
SS	48/161 (30%)	N.S.	$P = 0.0005$	41/115 (36%)	N.S.	$P = 0.002$	7/46 (15%)	N.S.	N.S.
IgG4 SS	86/221 (39%)	$P < 0.0001$		76/160 (48%)	$P < 0.0001$		10/61 (16%)	N.S.	
SL	24/173 (14%)			19/129 (15%)			5/44 (11%)		

SS, Sjogren's syndrome; IgG4 SS, IgG4-related sclerosing sialadenitis; SL, sialolithiasis; N.S., not significant.

VH usage patterns observed in SS and IgG4-related sclerosing sialadenitis were not specific. Most interestingly, VH clones were often unmutated in SS and IgG4-related sclerosing sialadenitis and the percentage ratios of unmutated/total clones were 30% and 39%, respectively. These rates were significantly higher than that of sialolithiasis clones (14%). In addition, the unmutated clones appeared to be derived mainly from the VH3 family because VH3 family clones were often unmutated in SS (36%) and IgG4-related sclerosing sialadenitis (48%), when compared with those in sialolithiasis (15%). In contrast, when non-VH3 family fragments were analysed, the unmutation ratios were uniformly low (11–16%) in all three lesions. Unfortunately, owing to the small number of clones analysed, we were unable to determine which fragment of the VH3 family contributed most to the higher rates of unmutated clones in SS and IgG4-related sclerosing sialadenitis cases.

Whether autoantibodies arise from somatic hypermutation of Ig genes or from less mutated or germline Ig genes has been controversial. Several studies have shown that autoantibodies are heavily mutated and back mutation of mutated human V genes to the germline sequences resulted in a loss of antigen binding [20–22]. However, other reports did not support these findings [23–25]. Some studies have shown a low rate of somatic mutation in autoantibodies of patients with SS [17, 26, 27]. In another study, an increased rate (19.6%) of unmutated clones was reported in the parotid gland specimen from a patient with SS [18]. In addition, VH gene analyses of non-Hodgkin lymphomas in patients with SS have shown that neoplastic B cell populations are often unmutated [14–28]. Our finding that B cells infiltrating inflammatory lesions of patients with SS possess less mutated VH genes is in line with these observations and supports the hypothesis that some germline or less mutated genes may play a role in the development of this autoimmune disease. Moreover, autoantibodies encoded by such genes fail to be deleted in patients with SS. IgG4-related sclerosing sialadenitis is a chronic inflammatory disorder characterized by a dense infiltration of IgG4-positive plasma cells. As treatment with steroids is very effective, an autoimmune mechanism is highly implicated in the aetiology of IgG4-related sclerosing sialadenitis. In this study, we showed that VH fragments of IgG4-related sclerosing sialadenitis and SS cases shared a common characteristics, a high rate of unmutated VH clones probably derived from the VH3 family. This finding suggests that an autoimmune mechanism similar to that of SS may also be responsible to the development of IgG4-related sclerosing sialadenitis.

In conclusion, we studied VH usage and VH somatic hypermutation in SS and IgG4-related sclerosing sialadenitis using sialolithiasis tissues as a control. The VH fragments, especially those of the VH3 family, were often unmutated when compared with those of the sialolithiasis cases. This finding will provide insight into the pathogenesis of SS and IgG4-related sclerosing sialadenitis.

Authors contributions

H. S., T. J., K. S, and H. I. designed research; H.S., F.O., and S.M. performed research; H.S., F. O., S. M., and H.I. analyzed data; and H. S. and H.I. wrote the paper.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Data S1 Sequence analysis of Sjogren's syndrome cases.

Data S2 Sequence analysis of IgG4-related sclerosing sialadenitis cases.

Data S3 Sequence analysis of sialolithiasis cases.

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Review Article

Double control systems for human T-cell leukemia virus type 1 by innate and acquired immunity

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Human T-cell leukemia virus type 1 (HTLV-1) is the causative retrovirus of adult T-cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). HTLV-1-specific T-cell responses elicit antitumor and antiviral effects in experimental models, and are considered to be one of the most important determinants of the disease manifestation, since they are activated in HAM/TSP but not in ATL patients. The combination of low T-cell responses and elevated HTLV-1 proviral loads are features of ATL, and are also observed in a subpopulation of HTLV-1 carriers at the asymptomatic stage, suggesting that these features may be underlying risk factors. These risks may potentially be reduced by vaccination to activate HTLV-1-specific T-cell responses. HAM/TSP and ATL patients also differ in their levels of HTLV-1 mRNA expression, which are generally low *in vivo* but slightly higher in HAM/TSP patients. Our recent study indicated that viral expression in HTLV-1-infected T-cells is suppressed by stromal cells in culture through type-I IFNs. The suppression was reversible after isolation from the stromal cells, mimicking a long-standing puzzling phenomenon in HTLV-1 infection where the viral expression is very low *in vivo* and rapidly induced *in vitro*. Collectively, HTLV-1 is controlled by both acquired and innate immunity *in vivo*: HTLV-1-specific T-cells survey infected cells, and IFNs suppress viral expression. Both effects would contribute to a reduction in viral pathogenesis, although they may potentially influence or conflict with one another. The presence of double control systems for HTLV-1 infection provides a new concept for understanding the pathogenesis of HTLV-1-mediated malignant and inflammatory diseases. (*Cancer Sci* 2011; 102: 670–676)

It has been three decades since the discovery of human T-cell leukemia virus type 1 (HTLV-1) as the causative retrovirus of adult T-cell leukemia (ATL).[1,2] ATL develops during middle age or later mainly in a small portion of vertically HTLV-1-infected populations.[3,4] HTLV-1 also causes HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) in another small population of infected individuals.[5,6] Some other inflammatory diseases such as uveitis and arthritis are also associated with HTLV-1 infection.[7,8] New therapeutic approaches such as hematopoietic stem cell transplantation (HSCT),[9,10] an antibody therapy targeting CCR4,[11] and antiviral therapy with interferon-alpha and zidovudin[12] partly improved the prognosis of ATL. However, ATL still shows high mortality, and HAM/TSP remains to be an intractable disease.

Enormous amounts of research findings have been accumulated regarding the virus-mediated pathogenesis. HTLV-1 Tax, a virus-encoded regulatory gene product, mediates cell activation, proliferation and resistance to apoptosis by transactivation through NF-κB, cAMP response element binding protein (CREB) and serum response factor (SRF), and by inactivation of tumor suppressors,[13–15] which would be involved in leukemogenesis and inflammation in HTLV-1 infection. Another minus-strand HTLV-1-encoded gene product, HTLV-1 basic leucine zipper factor (HBZ), is continuously expressed in infected cells *in vivo* regardless of the disease and may also be involved in the growth ability of infected cells.[16]

However, many unsolved questions still remain regarding the pathogenesis of HTLV-1 infection, for example, how the same virus causes totally different diseases such as ATL and HAM/TSP, why only small portions of HTLV-1-infected populations develop diseases, and why it takes more than 40 years to develop ATL. The answers to these questions would provide hints for predicting disease risks as well as aiding the development of prophylactic and therapeutic strategies.

HTLV-1-specific T-cell responses that contribute to antiviral and antitumor surveillance could be one of the most important determinants of the diseases. In fact, HTLV-1-specific T-cells are activated in HAM/TSP but not in ATL.[17–19] Oral HTLV-1 infection induces T-cell tolerance to HTLV-1 and increased proviral loads,[20,21] consistent with the epidemiological finding that vertical HTLV-1 infection is one of the risk factors for ATL.[3] Therefore, the individual status of HTLV-1-specific T-cell responses is expected to be an indicator of risk for ATL.[22] Although the pathological significance of HTLV-1-specific T-cells in HAM/TSP remains controversial,[23,24] advantages for HLA-A02-positive individuals in protection against HAM/TSP have been reported, and interpreted through the association of this HLA with strong CTL responses to a major epitope of HTLV-1 Tax.[25]

Elevation of proviral loads is also a risk factor for ATL. Given the fact that HTLV-1-specific CTLs have antiviral effects, these CTLs are likely to be one of the determinants of proviral loads.[26] However, proviral loads are also increased in HAM/TSP patients, and the correlations between proviral loads and HTLV-1-specific T-cell responses vary among studies,[27,28] suggesting the presence of additional factors for determining individual proviral loads.

Another curious finding in HTLV-1 infection is the scarcity of viral antigen expression in the peripheral blood, although the viral mRNA is barely expressed.[29] The transcription of HTLV-1 is mainly regulated by CRE-like repeats in the HTLV-1 LTR.[30] Involvement of inducible cAMP early repressor (ICER) and transducers of regulated CREB 2 (TORC2) in the inhibition of HTLV-1 transactivation has been suggested.[31,32] However, the mechanism involved in suppressing viral expression only *in vivo* has remained obscure. It is a paradox that HTLV-1 Tax contributes to the pathogenesis while Tax protein is undetectable *in vivo*. Expression of HBZ in the absence of Tax may partly

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explain the growth advantage of infected cells,[33] but not all of HTLV-1-mediated leukemogenesis. In addition, it does not make sense that Tax-specific T-cell responses are maintained if Tax is not expressed *in vivo*. The paradox will remain until the state of viral expression and the mechanisms for suppressing HTLV-1 expression *in vivo* are clarified.

We recently found that innate immune responses, especially type-I interferons (IFNs), suppress HTLV-1 expression.[34] This integrates the issue of viral expression and the host defense system against HTLV-1, which includes innate immunity as well as acquired immunity. The presence of double control systems explains some of the paradox in persistent HTLV-1 infection, and adds new aspects to the pathogenesis of HTLV-1-mediated diseases.

Control of HTLV-1 by HTLV-1-specific T-cell responses

Antitumor surveillance by HTLV-1-specific T-cells. $CD8^+$ HTLV-1-specific CTL responses are found in many HAM/TSP patients and asymptomatic carriers (AC), but rarely in ATL patients.[17–19,35,36] These CTLs kill HTLV-1-infected cells *in vitro*, and mainly recognize HTLV-1 Tax.[18,37] The HTLV-1 envelope is also a popular target, especially for $CD4^+$ CTLs.[38] Other viral antigens, including polymerase,[39] ROF (p12) and TOF (p30/p13),[40] and HBZ,[41] have also been shown to be targets of CTLs. Elimination of $CD8^+$ cells among PBMCs from HAM/TSP patients induces HTLV-1 expression during subsequent cell culture,[42] clearly indicating that $CD8^+$ HTLV-1-specific CTLs contribute to the control of HTLV-1-infected cells.

A series of animal model experiments indicated that HTLV-1-specific T-cell responses limit the expansion of HTLV-1-infected cells *in vivo*. Oral HTLV-1 infection induced insufficiency of HTLV-1-specific T-cell responses in rats, and the HTLV-1 proviral loads were inversely correlated with HTLV-1-specific T-cell responses.[21] Re-immunization of these rats with mitomycin C-treated HTLV-1-infected cells restored HTLV-1-specific T-cell responses and reduced the proviral loads[43] (Fig. 1). In another rat model of HTLV-1-induced tumors, the otherwise fatal HTLV-1-infected lymphomas in T-cell-deficient rats were eradicated by transfer of T-cells from syngeneic rats that had been vaccinated with a Tax-encoding DNA or peptides corresponding to a major epitope for Tax-specific CTLs.[44,45]

Recent clinical reports have indicated that HTLV-1-carrying recipients after liver transplantation developed ATL under the administration of immunosuppressants.[46,47] In contrast, Tax-specific CTL responses were strongly activated in some ATL patients who obtained complete remission after HSCT, but were not observed in the same patients before transplantation.[48] These findings suggest that HTLV-1-specific T-cells, including Tax-specific CTLs, play important roles in antitumor surveillance against HTLV-1 leukemogenesis.

Insufficient HTLV-1-specific T-cell responses as a potential risk for ATL. Most HTLV-1-infected individuals are asymptomatic, and only about 5% develop ATL and <1% develop HAM/TSP.[3,49] The epidemiological risk factors for ATL include vertical transmission and increases in the number of abnormal lymphocytes or HTLV-1 proviral loads.[3,50,51] HTLV-1 proviral loads are also elevated in HAM/TSP patients.[52]

Immunological studies have suggested that insufficiency in host T-cell responses against HTLV-1 might be another risk factor for ATL.[22] A small-scale survey measuring Tax protein-specific IFN-γ production revealed a wide variety in the strengths of HTLV-1-specific T-cell responses among HTLV-1 carriers.[53] The combinations of HTLV-1-specific T-cell responses and proviral loads categorize HTLV-1 carriers into the following four groups: (i) low proviral loads with HTLV-1-specific T-cell responses; (ii) elevated proviral loads with HTLV-1-specific T-cell responses; (iii) low proviral loads with low T-cell responses; and (iv) elevated proviral loads with low T-cell responses (Fig. 2).

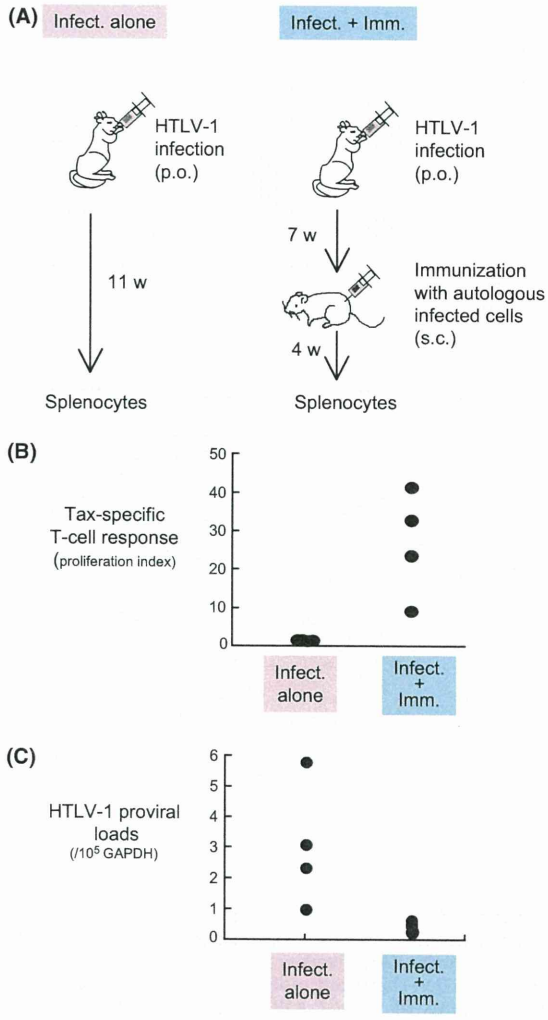

Fig. 1. Recovery of human T-cell leukemia virus type 1 (HTLV-1)-specific T-cell responses and reduction of proviral loads by re-immunization. Eight rats orally infected with HTLV-1 were divided into two groups. (A) One group was left untreated (Infect. alone) and the other was subcutaneously immunized with mitomycin C-treated HTLV-1-infected syngeneic rat T-cells (Infect. + Imm.) at 4 weeks. Spleen T-cells were harvested at 7 weeks after infection. (B,C) T-cells from the re-immunized rats (Infect. + Imm.) show elevated levels of Tax-specific T-cell proliferative responses (B) and lower proviral loads (C), compared with untreated rats (Infect. alone).[43]

Regarding these groups, ATL patients exhibit elevated proviral loads with low T-cell responses, while many, but not all, HAM/TSP patients show elevated proviral loads with high HTLV-1-specific T-cell responses. ACs are found in all four categories. It is noteworthy that small subgroups of ACs and smoldering ATL patients share a common feature with ATL patients. This indicates that the insufficiency of HTLV-1-specific T-cell responses is not merely the result of malignancy but is an underlying problem before the stage without apparent lymphoproliferation. Further follow-up studies are required to clarify whether the extent of the combination of elevated proviral loads with low T-cell responses could be a diagnostic indicator for risk of ATL.

Dissociation between proviral loads and T-cell responses. Although HTLV-1-specific T-cells have the potential to control infected cells, there are no clear correlations between

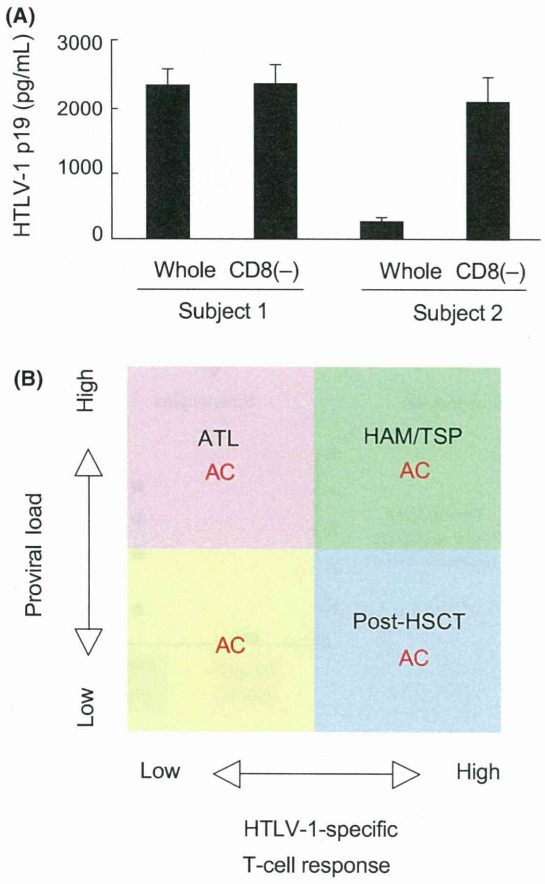

Fig. 2. Diversities in Tax-specific T-cell responses and dissociation with proviral loads in human T-cell leukemia virus type 1 (HTLV-1)-infected individuals. (A) Diversity in $CD8^+$ T-cell functions in two representative HTLV-1-infected individuals at the asymptomatic stage. Abundant amounts of HTLV-1 p19 were produced in PBMC cultures with or without $CD8^+$ T-cells in subject 1, but only after $CD8^+$ T-cell depletion in subject 2.[53] (B) A general image for the categories of HTLV-1-infected individuals at various stages according to the combinations of HTLV-1-specific T-cell responses (*x*-axis) and proviral loads (*y*-axis) is shown schematically. AC, asymptomatic carriers; ATL, adult T-cell leukemia; HAM/TSP, HTLV-1-associated myelopathy/tropical spastic paraparesis; HSCT, hematopoietic stem cell transplantation.

proviral loads and HTLV-1-specific T-cell responses among HTLV-1-infected individuals. This is not surprising because both the proviral loads and T-cell responses are high in HAM/TSP patients. The proviral loads may be negatively correlated with T-cell responses only within an individual but not among individuals. Several other reports have indicated various findings concerning this issue. For example, a study measuring IFN-γ-producing $CD8^+$ HTLV-1-specific CTLs indicated a positive correlation with proviral loads in HAM/TSP patients but not in ACs,[28] while a study evaluating $CD8^+$ CTL function by *ex vivo* clearance of infected cells showed negative correlations with low proviral loads within an AC or a HAM/TSP group,[42] and another study indicated an association of higher frequency of tetramer-binding Tax-specific CTLs with low proviral loads in ACs.[27] Such inconsistent results suggest the presence of certain other determinants of proviral loads in addition to HTLV-1-specific CTLs.

The HTLV-1 proviral loads reflect the number of infected cells in the peripheral blood. Expansion of HTLV-1-infected cells *in vivo* occurs through both *de novo* infection and proliferation of infected cells.[54] The number of $CD4^+$ $FoxP3^+$ cells,[55] the frequency of iNKT cells,[56] or MHC-I favorable for HBZ-specific T-cell responses[41] have been suggested to influence HTLV-1 proviral loads.

In HTLV-1-infected rats, however, the proviral loads are inversely correlated with HTLV-1-specific T-cell responses.[21] One reason for the discrepancy between humans and rats may be the genetic heterogeneity in humans. It appears that, under the homogeneous genetic background in the experimental rat system, the influence of insufficient HTLV-1-specific T-cell responses may appear more clearly than in humans, allowing *de novo* infection and proliferation of HTLV-1-infected cells *in vivo*. The dissociation of proviral loads and HTLV-1-specific T-cell responses in humans suggests that additional determinants of proviral loads may vary genetically among individuals. As described in the next section, we suppose that innate immunity could be a candidate for this effect.

Control of HTLV-1 by innate immunity

Status of HTLV-1 expression *in vivo*. Since HTLV-1-specific antibodies and T-cells are maintained in HTLV-1-infected individuals, viral expression must occur somewhere *in vivo*. This notion is further supported by the emergence of Tax-specific CTL responses in HTLV-1-uninfected donor-derived hematopoietic systems reconstituted in recipient ATL patients after HSCT.[48,57] However, HTLV-1 mRNA but not viral proteins are detectable in PBMCs freshly isolated from HTLV-1-infected individuals. The levels of HTLV-1 mRNA are higher in HAM/TSP patients than in ACs,[58] but viral proteins are still undetectable. Only a few reports have indicated HTLV-1 protein expression *in situ*.[59]

HTLV-1 expression in ATL cells immediately after isolation from the peripheral blood is very low, and becomes significantly induced after culture for some hours *in vitro*.[60,61] This phenomenon is observed in about one half of ATL patients regardless of the disease severity.[62] Viral induction after *in vitro* culture does not occur in the other one half of ATL patients, probably because of genetic and epigenetic changes in the viral genome.[63–65] Rapid induction of viral expression after *in vitro* culture has also been observed in PBMCs from HAM/TSP patients and ACs,[66] indicating that there must be a common mechanism for transiently suppressing HTLV-1 expression *in vivo* regardless of the diseases.

Suppression of HTLV-1 expression by type-I IFN responses. Recently, we found that type-I IFN responses are involved in the suppression of HTLV-1 expression.[34] When HTLV-1-infected T-cell line cells were co-cultured with stromal cells such as epithelial cells and fibroblasts, HTLV-1 mRNA and proteins were markedly decreased in HTLV-1-infected cells. Similarly, induction of HTLV-1 expression in cultures of primary ATL cells was also suppressed by co-culture with stromal cells. Type-I IFNs were involved in the stromal cell-mediated suppression of HTLV-1 expression, because it was partly neutralized by anti-IFN-α/β receptor antibodies. Since efficient HTLV-1 expression is dependent on transactivation of its own LTR by Tax protein,[30,67] limitation of this protein below a certain level will lead to the maintenance of HTLV-1 expression at low levels. Stromal cells reduced viral expression via type-I IFNs, but did not reduce cell growth and even supported it by unknown mechanisms.[34,68]

It has been reported that plasmacytoid dendritic cells (pDCs), a major producer of type-I IFNs, are susceptible to HTLV-1 infection.[69,70] In ATL patients, pDCs are decreased in number and also lack the ability to produce IFN-α.[69] A recent report indicated that pDCs generate type-I IFNs mainly through TLR7 recognition of HTLV-1 RNA.[71] The precise mechanisms of the HTLV-1-mediated IFN responses remain to be clarified.

In addition to recombinant IFN-α and IFN-β, recombinant IFN-γ was also capable of reducing HTLV-1 expression to

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lesser extents in HTLV-1-infected cell lines.[34,72] Participation of type-II IFN-producing cells other than stromal cells in HTLV-1 suppression *in vivo* is also conceivable.

Potential involvement of type-I IFNs in HTLV-1 suppression *in vivo*. In *in vitro* experiments, co-cultured stromal cells suppressed viral expression in HTLV-1-infected cells. Interestingly, when infected cells were re-isolated from the co-cultures, viral expression was restored to the original level over the following 48 h (Fig. 3).[34] This observation shows a striking similarity to the rapid induction of HTLV-1 expression in freshly isolated ATL cells after culture *in vitro*.

Involvement of type-I IFN responses in the suppression of HTLV-1 expression *in vivo* was confirmed using interferon regulatory factor-7-KO mice, which are deficient in most type-I IFN responses. Viral expression in HTLV-1-infected cells was significantly suppressed when the infected cells were intraperitoneally injected into WT mice but not into interferon regulatory factor-7-KO mice.[34]

It is speculated that the levels of viral expression in HTLV-1-infected lymphocytes may differ among various tissues depending upon the strength of IFN responses. Thus far, there is little information regarding HTLV-1 expression in various tissues. In transgenic mice with an HTLV-1 LTR-driven construct of the pX gene, expression of the transgene was only observed in limited organs including the central nervous system, eyes, salivary glands and joints.[73] It is intriguing that all of these tissues are involved in human inflammatory diseases related to HTLV-1 infection. Such coincidences suggest the involvement of HTLV-1 gene expression in the pathogenesis of these inflammatory diseases.

Double control of HTLV-1 by innate and acquire immunity

Relationship between acquired and innate immune control in HTLV-1 infection. At the primary infection, type-I IFNs generally play a critical role in limiting viral replication, and have positive effects on antigen presentation by activating DCs, inducing type-II IFN, and upregulating MHC-I, which subsequently augments T-cell responses.[74] However, the role of type-I IFNs in the chronic phase of viral infection may not always be positive. In HIV-1 infection, type-I IFNs may be a progressive factor for the disease by accelerating T-cell exhaustion.[75]

Suppression of HTLV-1 expression by type-I IFNs may reduce the efficacy of T-cell-mediated surveillance against HTLV-1-infected cells, because T-cells require viral proteins for recognition. On the contrary, if the IFN-mediated suppressive system is insufficient, HTLV-1-specific T-cell responses will be activated in response to viral antigens.

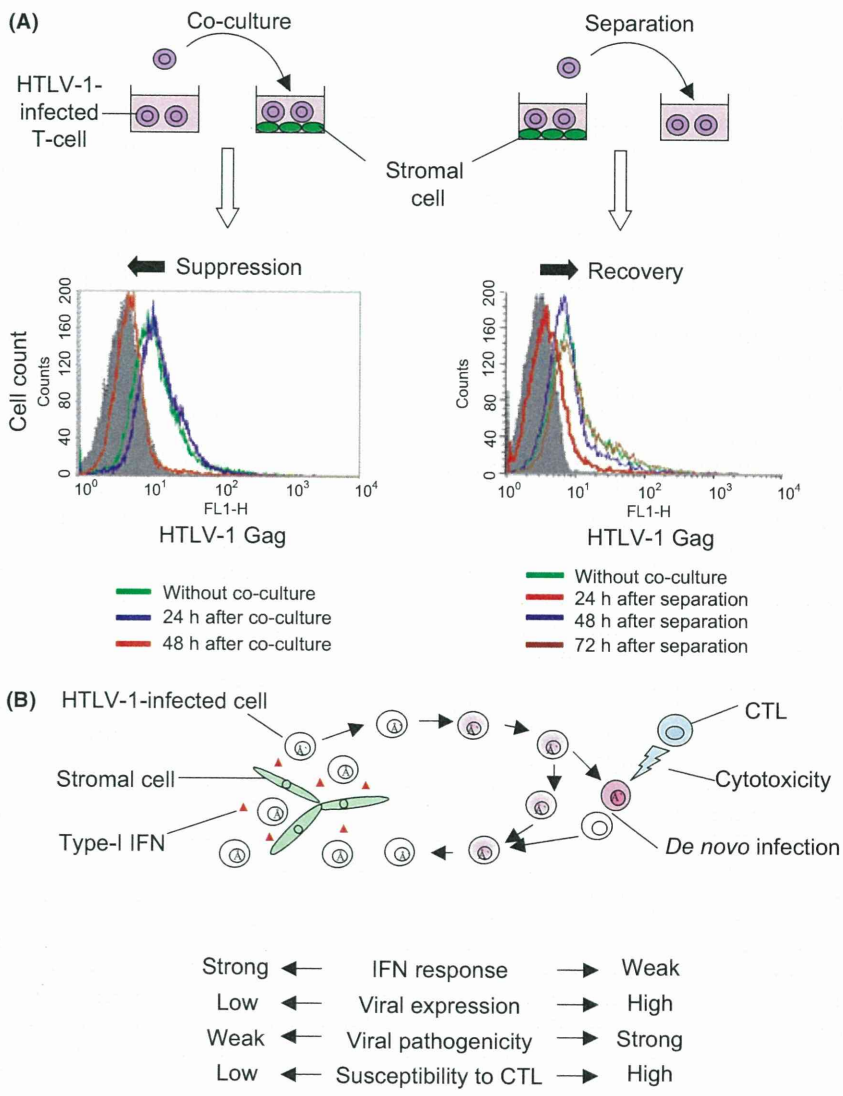

Fig. 3. Reversible suppression of human T-cell leukemia virus type 1 (HTLV-1) expression by innate immunity. (A) When IL-2-dependent HTLV-1-infected cells are co-cultured with 293T cells, intracellular HTLV-1 Gag proteins in the infected cells are decreased within 48 h (left panel). When the infected cells are re-isolated and further cultured on their own, Gag expression is recovered within 48 h (right panel).[34] (B) Scheme of the presumed status of HTLV-1-infected cells *in vivo*. Viral expression (indicated as pink) would be suppressed in tissues with strong IFN responses (left) and increased in tissues with weak IFN responses (right). CTL function, if any, is only effective upon viral expression, resulting in an infected cell reservoir without viral expression (left) and a T-cell surveillance system with low efficiency (right).

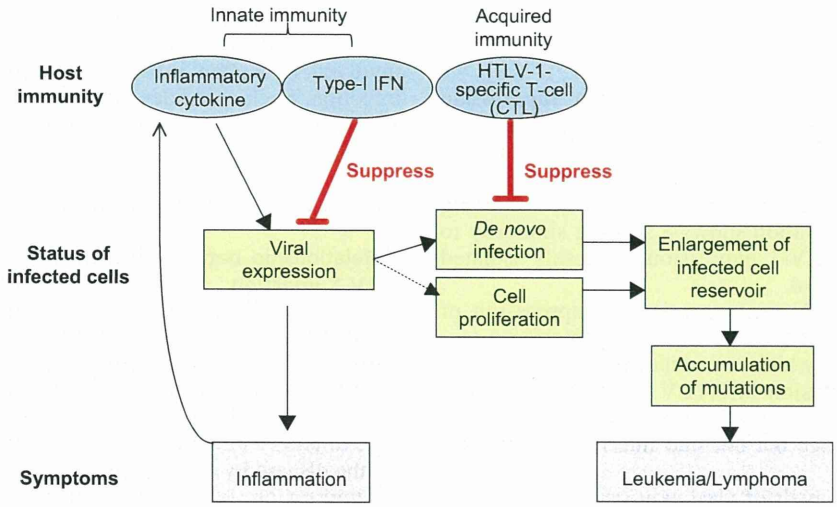

Fig. 4. Hypothetical relationships among the host immunity, status of human T-cell leukemia virus type 1 (HTLV-1)-infected cells and symptoms. HTLV-1-infected cells are controlled by at least two systems: type-I IFNs (innate immunity) and HTLV-1-specific T-cells (acquired immunity). The former suppress viral expression and the latter kill infected cells. An increase in viral expression would accelerate inflammation, increase the number of infected cells through *de novo* infection and activate HTLV-1-specific T-cells that determine an equilibrium level of proviral load within an individual. Viral expression may be a positive, but not absolute, factor for cell proliferation. When the viral expression is well controlled, the viral pathogenesis will proceed slowly, and may not be apparent until infected cell clones with a malignant phenotype finally emerge from the enlarged infected cell reservoir. Without proper T-cell responses, the emergence of such clones may occur earlier, because they would have more chance to survive.

The relationship between innate and acquired immunity may also differ among tissues. In tissues with strong IFN responses, viral expression in the infected cells would be suppressed and CTLs would ignore these cells. However, in tissues with weak IFN responses, infected cells would express viral antigens to be recognized by CTLs (Fig. 3). These presumptions can explain the status of HTLV-1-infected cells *in vivo*, which comprises a large reservoir of infected cells without viral expression and a low-efficiency surveillance system by CTLs that can only work on limited occasions.

Potential relationship between disease manifestation and innate and acquired host immunity in HTLV-1 infection. Although suppression of HTLV-1 expression may partly interfere with the efficacy of T-cell immunity, it may contribute to a slowing down of the Tax-mediated pathogenesis, tumorigenesis and inflammation (Fig. 4). In a rat model, shRNA-mediated suppression of Tax in HTLV-1-transformed cells rendered these cells resistant to Tax-specific CTLs but also reduced their ability for tumorigenesis *in vivo*.[76] Continuous suppression of HTLV-1 expression in humans may have a similar decelerating effect against Tax-mediated tumorigenesis. This might be a reason why it takes so long for ATL to develop. So long as the viral expression is well controlled, the viral pathogenesis may not be apparent until malignant cell clones finally come through the process of clonal evolution in the infected cell reservoir. Without proper T-cell responses, the emergence of such clones may occur earlier, because they would have more chance to survive.

HAM/TSP patients show elevated levels of viral expression for an unknown reason. Increased levels of inflammatory cytokines could be either a cause or a result of this phenomenon. The involvement of HTLV-1 proviral integration sites in transcription units in elevated viral expression has also been suggested.[77] An experimental rat model of HAM/TSP using a certain WKAH strain exhibits increased Tax mRNA expression in the spinal cord without T-cell infiltration,[78] suggesting that viral expression is a primary event while T-cell responses are not. Further studies revealed that this particular rat strain contains mutations in the promoter region of the IL-12 receptor, which potentially lead to reduced IFN-γ production in the spinal cord.[72] The associations of genetic factors related to the IFN system with HAM/TSP patients have remained obscure. Very recently, a gene expression profiling study indicated that expression of suppressor of cytokine signaling 1 (SOCS1) is upregulated in HAM/TSP patients and ACs, and is positively correlated with high HTLV-1 mRNA loads.[79]

Conclusions

HTLV-1 is controlled by both acquired and innate immunity. HTLV-1-specific T-cells contribute to antitumor surveillance, and type-I IFNs contribute to silencing viral expression. The presence of the double control systems with partial conflicts would explain some of the puzzles in HTLV-1 infection, such as the transient suppression of viral expression *in vivo*, apparently reciprocal occurrence of ATL and HAM/TSP, inconsistent correlations of proviral loads with T-cell responses, and a long incubation period.

Insufficient T-cell responses are regarded as a risk factor for ATL, and vaccines that augment HTLV-1-specific T-cell responses would be beneficial in reducing the risk in a subpopulation of HTLV-1 carriers exhibiting insufficient T-cell responses and elevated proviral loads.

Innate immune responses in HTLV-1 infection should be further investigated, because they could be another important determinant of disease manifestation and represent therapeutic targets in HTLV-1-related diseases.

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Clonal evolution of adult T-cell leukemia/lymphoma takes place in the lymph nodes

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Adult T-cell leukemia/lymphoma (ATLL) is the neoplasm caused by human T-cell leukemia virus type 1 (HTLV-1). We performed oligo-array comparative genomic hybridization (CGH) against paired samples comprising peripheral blood (PB) and lymph node (LN) samples from 13 patients with acute ATLL. We found that the genome profiles of the PB frequently differed from those of the LN samples. The results showed that 9 of 13 cases investigated had a log2 ratio imbalance among chromosomes, and that chromosome imbalances were more frequent in LN samples. Detailed analysis revealed that the imbalances were likely caused by the presence of multiple subclones in the LN samples. Five of 13 cases showed homozygous loss regions in PB samples, which were not found in the LN samples, indicating that tumors in the PB were derived from LN subclones in most cases. Southern blot analysis of TCRγ showed that these multiple subclones originated from a common clone. We concluded that in many ATLL cases, multiple subclones in the LNs originate from a common clone, and that a selected subclone among the LN subclones appears in the PB. (*Blood.* 2011;117(20):5473-5478)

Introduction

Adult T-cell leukemia/lymphoma (ATLL) is the neoplasm caused by human T-cell leukemia virus type 1 (HTLV-1). The disease is associated with poor prognosis due to drug resistance, the occurrence of opportunistic infections, a large tumor burden with multi-organ failure, and hypercalcemia. Shimoyama et al[1] classified ATLL into 4 subtypes: smoldering, chronic, lymphoma, and acute. It is also known that HTLV-1 infection alone does not facilitate the progress of infected $CD4^+$ T cells to fully malignant ATLL cells. Therefore, the search for genes involved in ATLL development and for the specific genes involved in each ATLL type has been actively pursued, albeit with limited success. ATLL-specific chromosomal abnormalities have yet to be found; however, a frequent abnormality found in ATLL is 14q11, which has also been found in other types of T-cell malignancies.[2,3] HTLV-1 provirus integration sites have also been extensively sought, and the sites identified were found to be randomly located. Investigations relying on G-band and fluorescence in situ hybridization analyses have not been fruitful in providing a detailed delineation of the genomic aberrations involved.[4] The use of high-resolution, array-based comparative genomic hybridization (CGH) for comprehensive chromosome analysis should prove useful in the search for genomic aberrations. We showed previously that acute and lymphoma ATLL types possess distinct genomic profiles, as determined by bacterial artificial chromosome array CGH.[5] It should be noted, however, that when lymphoma-type ATLL progresses to manifest more than 2% flower cells in the peripheral blood (PB), it is then classified as the acute type. We set out to analyze the genomic aberrations of acute-type ATLL with paired PB and lymph node (LN) samples in more detail by oligo-array CGH.

An important factor in the diagnosis of ATLL is the identification of monoclonal integration of HTLV-1. It has been reported that the same HTLV-1–infected clone was detected over several years in a chronic-type ATLL patient.[6,7] These types of HTLV-1–infected $CD4^+$ T lymphocytes are believed to accumulate various changes during an extensive latency period of over 50 years.[8] Alterations in genomic copy number represent one example of the type of accumulated genomic changes that can occur. In the present study, we performed high-resolution oligo-array CGH (Agilent Technologies) using a 44 000-probe set against paired samples obtained from the PB and LNs of 13 patients with acute-type ATLL.

Methods

ATLL patients and cell lines

We conducted a survey of genomic profiles by examination of PB and LN samples taken from 13 patients with acute-type ATLL. Paired samples were collected from each patient within 14 days of diagnosis. The PB and LN samples, together with clinical data, were obtained from 13 patients under a protocol approved by the institutional review board of the Aichi Cancer Center. Informed consent was provided according to the Declaration of Helsinki. Patients were diagnosed from those hospitalized between 1988 and 2010 at Imamura-Bunin Hospital and Nagasaki University School of Medicine. The diagnosis of ATLL was based on clinical features, hematologic characteristics, immunophenotype, and the presence of serum antibodies to ATLL-associated antigens. The median age of the patients was

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