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—九州血液疾患治療グループ（K-HOT）アンケート調査—

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はじめに

成人T細胞白血病リンパ腫（ATL）はhuman T-lymphotropic virus type 1（HTLV-1）が原因で発症し，化学療法での治療成績は極めて不良である[1)-3)]。近年同種造血幹細胞移植（allogeneic hematopoietic stem cell transplantation：allo-HSCT）でATL患者の予後が改善する可能性が報告され[4)5)]，allo-HSCT施行例が急速に増加しつつある。しかしながらATLに対するallo-HSCTのprospective studyの報告は厚生労働省・がん克服研究班（岡村純班長）の骨髄非破壊的同種末梢血幹細胞移植術の検討[6)]を除いてはなく，その適応やドナーの選択については全く定まっていない。せめてATL多発地域である九州地区の血液専門病院でのATLに対するallo-HSCTについての現状を把握する必要があると考えた。

今回，九州血液疾患治療グループ（K-HOT）参加施設におけるATLに対するallo-HSCTの適応基準についてアンケート調査を実施したので報告する。

Ⅰ．対象および方法

アンケート調査は多選択肢，一部記述式で，K-HOT参加施設の各責任者にアンケートを郵送し，回答を求めた。アンケートは(1)適応年齢，(2)臨床病型，(3)寛解状態，(4)病勢の安定，(5)移植時期，(6)ドナー及び幹細胞源，(7)前処置，(8)graft-versus-host disease（GVHD）の予防，(9)HTLV-1キャリアのドナーとしての適格性について調査した。

Ⅱ．結果

1．回収率と施設の背景

K-HOT参加23施設中20施設（87.0%）より回答が得られた。回答の得られた20施設中同種移植を実施している施設が18施設，自家移植のみが1施設，造血細胞移植を実施していない施設が1施設であった。

2．同種移植の適応年齢

骨髄破壊的移植の適応は50歳未満6施設，55歳未満10施設，60歳未満4施設であった。一方，骨

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⑱琉球大学医学部第二内科　⑲天草中央病院内科
⑳和歌山県立医科大学血液内科
㉑福岡大学病院血液・糖尿病内科

髄非破壊的移植の適応は50歳以上70歳未満8施設，55歳以上70歳未満9施設，60歳以上70歳未満3施設であった。

3. 臨床病型

急性型・リンパ腫型に限るが4施設，急性型・リンパ腫型に加え慢性型の予後不良因子を有するものまで含めるが8施設，急性型・リンパ腫型と慢性型の急性転化をした例を含めるが8施設であった。

4. 寛解状態

完全寛解のみが0施設，完全寛解と部分寛解が3施設，完全寛解と病勢が安定している部分寛解が3施設，完全寛解と部分寛解のみでなく病勢が安定していれば非寛解も含めるが14施設であった。

5. 病勢の安定した非寛解とは何を意味するか（複数選択可）

高カルシウム血症がない10施設，血清LDHが正常の2倍以下である7施設，3ヵ月の生存が期待できる16施設，全身状態(Performance status：PS)がよい6施設，リンパ節が消失2施設，中枢神経浸潤がない1施設であった。

6. 移植時期

化学療法開始後3～6ヵ月でできる限り完全寛解で実施するが7施設，完全寛解が得られなくてもできる限り6ヵ月以内で実施するが15施設，できる限り完全寛解を目指すが，得られない場合は移植を断念するが1施設であった。

7. ドナー及び幹細胞源について

1）HLAの一致度

HLA一致の血縁ドナーのみを対象とする施設は1施設のみで，HLA一致なら血縁・非血縁を問わない施設が6施設であった。また血縁では一座不一致までを対象とする施設が8施設で，血縁・非血縁ともに一座不一致までを対象とする施設が8施設であった。その他として一座不一致はDRのみとするが1施設，血縁のみ二座不一致までが1施設，non-inherited maternal antigen (NIMA) 不一致まで対象とするが1施設であった。

2）臍帯血移植

臍帯血移植は考慮しないが4施設，血縁・非血縁のHLA一致のドナーがいない場合や間に合わない場合に考慮するが16施設，このうち病勢が安定していることを条件にしている施設が6施設，細胞数が2×10^7/kg以上あることを条件にしている施設が12施設であった。

3）NIMA不一致同胞・子母間移植

NIMA不一致同胞・子母間移植は考慮しないが6施設，血縁・非血縁のHLA一致のドナーがいない場合や間に合わない場合に考慮するが14施設，このうちhigh risk症例に限るが2施設，マイクロキメリズムの証明されているものに限るが1施設であった。

4）血縁移植の幹細胞について

Peripheral blood stem cell (PBSC) がよいが11施設，bone marrow (BM) がよいが0施設，完全寛解ならBMで部分寛解や非寛解ではPBSCがよいが1施設，その他としてドナーの意向が2施設，症例毎が1施設，どちらでもよいが1施設，わからないが4施設であった。

8. 骨髄破壊的移植の前処置について

1）全身放射線照射（total body irradiation：TBI）

すべての同種移植でTBIを必須とするが8施設，臍帯血と不一致移植のみTBIを必須とするが3施設，非血縁（臍帯血を除く）の場合はできるだけTBIを行うが4施設，その他として不要やできるだけなしが3施設，症例毎に考慮するが2施設であった。

2）化学療法

Busulfan (BU)/cyclophosphamide (CY) もしくはCY/TBIでよいが12施設，etoposideやcytosine arabinosideをできるだけ含むが2施設，ATLの病勢に応じて前処置の薬剤を考慮するが6施設，不明が1施設であった。

9. GVHDの予防

1）血縁の一致ドナーからの移植

Cyclosporin A (CsA) + short term methotrexate (sMTX) とするが18施設，CsA単独とするが0施設，病勢に応じて免疫抑制剤を調整するが0施設，最良の方法がわからないが2施設であった。

2）非血縁（臍帯血を含む）及び不一致の移植

CsA+sMTXとする5施設，tacrolimus (FK506) + sMTXとするが13施設，CsAもしくはFK506単独でよいが2施設，その他最良の方法がわからないが2施設であった。

10. HTLV-1キャリアドナー

ドナーとして不適格であるが0施設，HTLV-1のモノクローナルやオリゴクローナルな増殖がなければ適格であるが17施設，HTLV-1プロウイルス量まで参考にして決めるが1施設，ATLの発

表 1　ATLの同種造血幹細胞移植の適応

1.	適応年齢 骨髄破壊的移植：55歳未満 骨髄非破壊的移植：50歳/55歳以上70歳未満
2.	臨床病型 急性型（急性転化を含む）・リンパ腫型
3.	寛解状態 完全寛解・部分寛解・病勢の安定している非寛解
4.	ドナーについて 血縁・非血縁ともにHLA完全一致もしくは一座不一致
5.	移植時期 化学療法開始後6ヵ月以内
6.	臍帯血移植・NIMA不一致移植・子母間移植 血縁・非血縁のHLA一致のドナーがいない場合や時間的に間に合わない場合
7.	前処置 TBI/CYまたはBU/CY
8.	GVHDの予防 血縁のHLA一致：CsA＋sMTX 非血縁または血縁不一致：FK506＋sMTX
9.	HTLV-1キャリアドナーの適格性 HTLV-1プロウイルスのモノクローナルやオリゴクローナルな組み込みがない

NIMA：non-inherited maternal antigen, TBI：total body irradiation, CY：cyclophosphamide, BU：busulfan, GVHD：graft versus host disease, CsA：cyclosporin A, sMTX：short term of methotrexate, FK506：tacrolimus, HTLV-1：human T-lymphotropic virus type 1.

症がなければドナーとして適格であるが2施設であった。

Ⅲ. 考　　察

K-HOT参加施設でのATLのallo-HSCTの適応基準についての現状を調査報告した。K-HOT参加施設はATLの多発地域であり多数のATL患者の診断治療を実際に行っている施設である。K-HOT参加施設の2000年2月からの約3年間に新たに診断した血液疾患2,908例中ATLが269例と約10%を占めていた[7]。これらK-HOT参加施設でのATLに対するallo-HSCTの適応基準の現状を把握することは非常に重要なことである。調査結果をまとめると適応年齢は骨髄破壊的移植では55歳未満が10施設と最も多かった（表1）。骨髄非破壊的移植では50歳以上70歳未満と55歳以上70歳未満がほぼ同数であった。骨髄非破壊的移植の安全性と有効性が確立されると骨髄非破壊的移植がより若年者にも適応されてくると思われる。臨床病型では急性型とリンパ腫型に加え予後不良因子を含む慢性型や慢性型の急性転化した例を含めるとする施設が多かった。しかしながら予後不良因子を有する慢性型の中には化学療法の適応にならない例も一部含まれており，慢性型ATLの同種移植の適応については現時点では議論の分かれるところであろう。寛解状態は完全寛解と部分寛解のみでなく病勢が安定していれば非寛解例も含めるとする施設が大部分を占めた。完全寛解例をallo-HSCTの適応に含めることに異論はみられるが，除外するエビデンスもない。今後，前方視的臨床研究にて結論を出すべきであろう。ATLでの同種移植の適応基準は，病勢の安定している非寛解期を適応に含めること以外は，他の造血器腫瘍の同種移植の適応とほぼ同じであると考えられる。ATLのallo-HSCTにおいては移植後にGVHDのみられなかった例に再発が多いとの報告[5)8)]やgraft-versus（GV）-tax response[9)]，GV-ATL効果が臨床的にみられた報告[10)11)]もあり，GV-ATL効果の存在が強く示唆されている。また，多くの施設は移植前の寛解状態にのみ重点を置くのではなく，患者の全身状態（PS）や最低3ヵ月の生存の確保が重要であると考えている。FukushimaらもATLの同種移植では，寛解・非寛解などの寛解状態よりもPSが重要であると報告している[11)]。血縁移植の幹細胞源についてPBSCがよいが11施設を占めていたが，他の疾患のallo-HSCTにおいてもPBSCTがよいか，BMTがよいかの結論は出ていない。Granulocyte-colony stimulating factor（G-CSF）はATL細胞を増殖させる可能性があることが報告されており[12)]，HTLV-1キャリアにおいてはPBSCTよりもBMTを推奨する考え方もある。しかし，HTLV-1キャリアにおいてG-CSFがどのような影響を及ぼすのかについては明らかではなく，今後の課題と思われる。TBIについては8施設が必要と答え意見が分かれたが，これはATLに対する前処置のあり方についてより強力にすべきか，より軽くすべきかの点で施設ごとに考え方に差があることのみでなく，骨髄破壊的移植と骨髄非破壊的移植に分けて回答を求めなかったこと，またTBIの照射量について言及しなかったことに起因しているものと思われる。

次に，ATLの血縁者間移植ではHTLV-1キャリアをドナーとして適格とするかどうかが問題である。ATL患者の同胞では約6割がHTLV-1キャリアであるとの報告[13)14)]もあり，キャリアということだけで不適格とすれば血縁者におけるドナー候補者はさらに制限されることになる。しかし一方では，ATLの家族内発症の問題もある[15)]。

野村らは7人兄弟で戦死した1名を除いた6名全員がATLで死亡した家系を報告しており[16]，またHTLV-1キャリアの血縁ドナーより同種移植を施行後ドナー由来の細胞からATLが発症した報告もある[17]。今回のアンケート結果でもHTLV-1キャリアをドナーとして適格とするかどうかは，サザンブロット検査でモノクローナルやオリゴクローナルなHTLV-1の組み込みがないことが必要であるとほぼ一致した回答が得られている。将来ATL細胞の遺伝子解析や血中HTLV-1プロウイルス量の測定などにより，ATLの発症機構の解明や発症高危険群が同定されれば，HTLV-1キャリアのドナーとしての適格性の判断が容易になるであろう。

ATLのallo-HSCTのK-HOT施設での適応について述べたが，ATLのallo-HSCTを実施するにあたって以下にあげる種々の問題がある。(1) allo-SCTまでの化学療法をどうするか，(2)中枢神経浸潤に対する対策（化学療法中の中枢神経浸潤に対する予防，前処置中や直前に抗腫瘍剤の髄腔内投与を施行するかどうか），(3)感染症対策，(4)HTLV-1キャリアドナーに対するG-CSFの使用の安全性（G-CSFはATL細胞を増殖させる可能性があるとの報告[12]があり，HTLV-1キャリアの感染細胞の異常増殖を引き起こさないかどうか），(5)GV-ATL効果の解明，(6)家族内にHTLV-1キャリアの存在（キャリアからのATL発症やキャリア自身のドナーとしての適格性など複雑な問題をかかえるATLの血縁者間allo-HSCT実施するにあたり，患者及び家族に対するinformed consentの方法をどうするか）など解明や解決すべき問題が多数残っている。

いずれにしてもATLに対するallo-HSCTの前方視的治療研究の報告は，骨髄非破壊的移植のPhase I study[6]を除いてはない。まずHLAの一致した血縁者間及び非血縁者間allo-HSCTにおいて，よいデザインで計画されたPhase II studyの成績が明らかにされることが必要である。NIMA不一致・子母間移植や臍帯血移植などの実験的移植は当科の解析結果では長期生存が非常に少なかった[18]。したがって，ATLの予後が極めて不良であることが予想される症例で，血縁の完全一致や一座不一致のドナーがいない場合やATLの病勢のため非血縁移植の間に合わない例に限って，十分な臨床経験のある施設においてプロトコール研究として評価されるべきである。これらの成績が示されてはじめて適応ガイドラインの作成が可能なものとなるであろう。

結　　語

ATLにおけるallo-HSCTのエビデンスは乏しく絶対適応もまだ定まっていない。今回の調査研究は，ATL多発地域における移植実施施設でのATLのallo-HSCTに対する一定の考え方が明らかにされ，プロトコール作成や共同研究の基礎資料として有用である。また，今後これらをもとに実施されたallo-HSCT症例を詳細に解析し，ATLに対するallo-HSCTの適応基準を作成する必要がある。

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Potential immunogenicity of adult T cell leukemia cells *in vivo*

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Experimental vaccines targeting human T cell leukemia virus type-I (HTLV-I) Tax have been demonstrated in a rat model of HTLV-I-induced lymphomas. However, the scarcity of HTLV-I-expression and the presence of defective HTLV-I-proviruses in adult T cell leukemia (ATL) cells have raised controversy about the therapeutic potential of HTLV-I-targeted immunotherapy in humans. We investigated the expression of HTLV-I antigens in fresh ATL cells by using both *in vitro* and *in vivo* assays. In flow cytometric analysis, we found that 3 of 5 acute-type and six of fifteen chronic-type ATL patients tested showed significant induction of HTLV-I Tax and Gag in their ATL cells in a 1-day culture. Concomitantly with HTLV-I-expression, these ATL cells expressed co-stimulatory molecules such as CD80, CD86 and OX40, and showed elevated levels of antigenicity against allogeneic T cells and HTLV-I Tax-specific cytotoxic T-lymphocytes (CTL). Representative CTL epitopes restricted by HLA-A2 or A24 were conserved in 4 of 5 acute-type ATL patients tested. Furthermore, spleen T cells from rats, which had been subcutaneously inoculated with formalin-fixed uncultured ATL cells, exhibited a strong interferon gamma-producing helper T cell responses specific for HTLV-I Tax-expressing cells. Our study indicated that ATL cells from about half the patients tested readily express HTLV-I antigens including Tax *in vitro*, and that ATL cells express sufficient amounts of Tax or Tax-induced antigens to evoke specific T cell responses *in vivo*.

Key words: cancer vaccine; human T cell leukemia virus type-I (HTLV-I); viral expression; co-stimulatory molecules; T cell immune response

Human T cell leukemia virus type-I (HTLV-I) is etiologically linked to adult T cell leukemia (ATL).[1–3] It is estimated that about 1 million people are infected with HTLV-I in Japan and 1–5% of infected subjects develop ATL.[4,5] Most other HTLV-I-carriers are asymptomatic throughout their lives and another small fraction of HTLV-I-carriers develop a chronic progressive neurological disorder termed HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP)[6,7] and other inflammatory disorders. Once patients develop acute-type ATL, leukemic cells resist anti-tumor chemotherapy, and the median survival time is 6.2 months.[8] Allogeneic hematopoietic stem cell transplantation (HSCT) has been applied recently in acute ATL patients and successful efficacy was obtained in some cases.[9,10] These effects may be attributed to a graft *vs.* leukemia reaction mediated by the donor-derived T cell immunity. There is also, however, a risk of graft *vs.* host reaction and its undesirable side effects are sometimes lethal. On this account, further improvement or new approaches are required for ATL treatment.

The precise mechanisms of HTLV-I-related diseases are not fully understood. HTLV-I viral protein Tax transactivates and interacts with many cellular proteins that regulate or dysregulate cell growth,[11] partly accounting for the mechanisms of HTLV-I-induced leukemogenesis.

In a rat model of HTLV-I-infected T cell lymphomas, uncontrollable expansion of tumor cells was highly associated with a functional defect or suppression of HTLV-I-specific T cell immunity including cytotoxic T lymphocytes (CTL).[12,13] Vaccination with autologous HTLV-I-infected cells,[12] Tax-encoding DNA,[14] or oligopeptides corresponding to a CTL-epitope[15] elicited anti-tumor effects in this model. HTLV-I Tax serves as an immunodominant target antigen for HTLV-I-specific CTL not only in rats but also in humans.[16,17] HTLV-I-specific CTL have been detected in the peripheral blood of HTLV-I-infected individuals[18] and can be induced from healthy carriers and HAM/TSP.[16,19,20] HTLV-I-specific CTL, however, is induced infrequently from ATL patients.[21,22] Moreover, Tax-specific CTL are capable of killing short-term cultured ATL cells.[22,23] These observations indicated that immunotherapy directed against Tax might be effective for ATL.

It is controversial, however, whether HTLV-I-specific immunotherapy has any therapeutic advantages for ATL patients with advanced disease because of the scarcity of HTLV-I-expression in ATL cells. ATL cells sometimes contain mutations and deletions in HTLV-I proviral genome,[24,25] and the ATL cells may not be able to express Tax. It is also known that viral expression in freshly isolated peripheral ATL cells is transiently suppressed.[26–28]

The reasons for insufficient HTLV-I-specific T cell response in ATL patients are also unclear. We found recently that a strong Tax-specific CTL response was induced in ATL patients after HSCT from HLA-identical donors,[29] indicating that the immune insufficiency in these patients before transplantation was not HLA-related. Pique *et al.*[30] reported that HTLV-I-specific CTL do exist in ATL patients but insufficiently expand. This suggests involvement of some immune suppression or tolerance. Alternatively, the levels of viral expression in ATL cells may be too low to evoke T cell immunity *in vivo*.

Because these cells may be a vaccine candidate, we investigated HTLV-I-expression of fresh ATL cells from 5 acute-type

Abbreviations: ATL, adult T cell leukemia; CTL, cytotoxic T lymphocytes; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FSC, forward scatter; HAM/TSP, HTLV-I-associated myelopathy/tropical spastic paraparesis; HLA, human leukocyte antigen; HSCT, hematopoietic stem cell transplantation; HTLV-I, human T cell leukemia virus type-I; IFN-γ, interferon-gamma; IL, interleukin; LTR, long terminal repeat; mAb, monoclonal antibody; MHC-II; Class II major histocompatibility complex; MLR, mixed lymphocyte reaction; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PE, phycoerythrin; PHA, phytohemagglutinin; SSC, side scatter.

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and 15 chronic-type ATL patients to determine whether ATL cells themselves can be immunogenic and evoke HTLV-I-specific T cell response. We demonstrated that in nearly 50% of the ATL patients tested HTLV-I Tax was inducible after short-term culture. Nucleotide sequences of HTLV-I *tax* at representative CTL epitopes in these ATL cells were mostly conserved. Interestingly, rats inoculated with formalin-treated uncultured ATL cells successfully developed helper T cell responses specific for Tax-expressing cells *in vivo*, indicating that ATL cells may express a small but sufficient amount of HTLV-I antigens for T cell response *in vivo*. Our findings suggest that ATL cases may be divided into 2 groups depending on the ability to express HTLV-I antigens and in nearly 50% the cases of ATL patients, ATL cells may potentially be recognized by HTLV-I-specific T cells *in vivo*.

Material and methods

Patients and PBMC preparation

Heparinized peripheral blood samples were donated under informed consent from 19 patients diagnosed as acute-type or chronic-type ATL at Ryukyu University Hospital, Imamura Bun-in Hospital in Kagoshima, and Nagasaki University Hospital and from uninfected healthy volunteers. The clinical status of these patients is summarized in Table I. The diagnosis and clinical subtype of ATL were made according to Shimoyama's criteria.[8] The ATL patients did not receive any chemotherapy when tested. Two samples from Patient 1 were used in our study. The first sample (1-i) was taken at a chronic phase and the other (1-vi) was taken at the acute crisis after an 18-month interval. Otherwise, one sample per patient was used. PBMC were isolated by using Ficoll-Paque (Amersham Pharmacia, Uppsala, Sweden) density centrifugation and cryopreserved in liquid nitrogen until use.

Animals

Inbred female F344/N Jcl-rnu/+ rats (F344 n/+; 4-week-old) were purchased from Clea Japan, Inc. (Tokyo, Japan). Rats were treated under the experimental protocol of the Animal Care Committee of our university.

Cell lines

HTLV-I-negative human T cell line Molt-4[31] and HTLV-I-producing human T cell line MT-2[32] were maintained in 10% heat-inactivated FBS (Sigma, St. Louis, MO), 100 U/mL of penicillin, 100 μg/mL of streptomycin in RPMI 1640 medium (Sigma) (10% FBS-RPMI).

ILT-Hod,[33] an IL-2-dependent HTLV-I-infected human T cell line was maintained in the presence of 10 U/mL of recombinant human IL-2 (rhIL-2; Shionogi Co., Osaka, Japan) in 10% FBS-RPMI. In addition, 2 other IL-15-dependent HTLV-I-infected human T cell lines, ILT-79 and ILT-85, were established from ATL Patients 79 and 85, respectively. To establish these lines, a CD4-positive cell-enriched fraction negatively separated from PBMC by using Dynabeads M-450 CD8 (Dynal, Oslo, Norway) and Dynabeads M-450 CD19 (Dynal) was stimulated with 1 μg/mL of phytohemagglutinin (PHA-p; Difco Laboratories, Detroit, MI) for 24 hr, washed and cultured in 10% FBS-RPMI containing 10 ng/mL of rhIL-15 (Sigma) for 1–3 months.

HLA-A24-restricted HTLV-I Tax-specific $CD8^+$ CTL line was induced from PHA-p-stimulated PBMC of a post-HSCT ATL patient by repeated stimulation with formalin-fixed autologous HTLV-I-infected cells established before the HSCT.[29] The CTL line was maintained in the presence of 100 U/mL of rhIL-2 with periodical stimulation with formalin-fixed autologous HTLV-I-infected cells at 10–14 day intervals.

HTLV-I-infected rat T cell line, FPM1,[12] derived from an F344 n/+ rat, were cultured in 10% FBS-RPMI. G14[14] is IL-2-dependent HTLV-I negative $CD8^+$ T cell line established from a F344 n/+ rat. G14-Tax[14] is a stable transfectant of G14 with HTLV-I Tax-expressing plasmids. G14 and G14-Tax were maintained in 10% FBS-RPMI containing 5.5×10^{-5} M of 2-mercaptoethanol and 10 U/mL of rhIL-2.

Monoclonal antibodies

To detect intracellular HTLV-I antigens, mouse monoclonal antibodies (mAbs), Lt-4 (anti-p40 Tax, mouse IgG3),[34] NOR-1 (anti-p24 and p53 Gag; mouse IgG1),[35] GIN-7 (anti-p19, p28 and p53 Gag; mouse IgG2b)[35] and biotinylated GIN-7 were used.

For cell surface characterization, fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated mouse anti-human CD4, CD8, CD25, CD40, CD40L, CD86, OX40, HLA-A, B, C, HLA-DR (IgG1; BD Pharmingen Co., San Diego, CA), CD80 (IgG1; Immunotech, Marseille, France) and OX40L (TAG-34, IgG1)[36] mAbs were used. In addition, FITC-conjugated mouse anti-rat CD4 and PE-conjugated mouse anti-rat CD8 mAbs (IgG1; BD Pharmingen Co.) were used.

TABLE I – CLINICAL STATUS OF ATL PATIENTS TESTED

Patient ID	Age	Gender	Type of ATL	WBC number/ μL	Mononuclear cells/ WBC (%)	Abnormal lymphocytes/ WBC (%)
#1-vi[1]	60	F	Acute	42,000	63	49
#22	38	M	Acute	16,100	50	35
#80	39	M	Acute	141,000	>95	91
#85	77	M	Acute	67,400	74	67
#91	66	F	Acute	27,200	89	61
#1-i[1]	58	F	Chronic	21,500	87	55
#5	72	M	Chronic	12,200	64	25
#6	70	F	Chronic	14,400	71	33
#7	62	F	Chronic	8,300	69	34
#8	60	F	Chronic	17,400	70	47
#20	64	M	Chronic	14,700	48	28
#23	68	M	Chronic	14,200	74	58
#29	58	M	Chronic	6,000	59	21
#42	54	F	Chronic	8,800	48	15
#54	63	F	Chronic	10,700	73	43
#69	54	F	Chronic	13,300	68	36
#79	63	F	Chronic	19,500	64	6
#89	50	F	Chronic	28,000	41	27
#90	53	M	Chronic	7,700	30	8
#92	66	F	Chronic	16,900	83	76

[1]Pt. #1-i and Pt. #1-vi are the identical individual with an initial diagnosis of chronic ATL, whose diagnosis changed to acute ATL associated with elevated levels of serum LDH after 18 months.

Intracellular and surface staining and flow cytometric analysis

For intracellular HTLV-I-staining, cells were fixed with 1% paraformaldehyde in PBS containing 20 μg/mL of lysolecithin (Sigma) for 2 min at room temperature. The cells were then centrifuged and resuspended in cold methanol. After incubation for 15 min at 4°C, the cells were centrifuged and incubated in 0.1% Triton-X in PBS for 5 min at 4°C. The cells were then washed with PBS containing 1% FBS and 0.1% NaN_3 (staining buffer), and incubated with mouse mAbs to HTLV-I antigens or BALB/c control ascites, and subsequently with FITC-conjugated goat anti-mouse IgG + IgM mAbs (Immunotech) for 30 min at room temperature. The optimal concentrations of these mAbs were determined before use. Cells were washed twice, fixed with 1% formalin in PBS and analyzed using a flow cytometer (FACS Calibur, Becton Dickinson, San Jose, CA). Live cells were gated based on a pattern of SSC and FSC for approximately 1×10^4 cells.

An alternative permeabilizing method using saponin was also employed for intracellular staining. Briefly, cells were fixed with 4% formalin in PBS, then permeabilized with 0.5% saponin (Sigma) in staining buffer for 10 min at room temperature. Permeabilized cells were further incubated with mAbs to HTLV-I antigens as described above.

For surface staining, cells washed and stained with FITC- or PE-conjugated mAbs and appropriate isotype control mAbs. Cells were further stained with 7-ADD (BD Pharmingen Co.) and stained cells were gated out on FACS analysis to eliminate dead cells.

For two-color analysis of intracellular and cell surface antigens, cells were stained with FITC-conjugated mouse anti-human mAbs (CD80, CD86, OX40), fixed and permeabilized by saponin treatment. Permeabilized cells were further stained with biotinylated GIN-7, and subsequently with Cy-chrome streptavidin (BD Pharmingen Co.). After extensive washing, the cells were subjected to two-color flow cytometry.

Long PCR and nucleotide sequences

Genomic DNA was prepared from PBMC by sodium dodecyl sulfate-proteinase K digestion, followed by phenol-chloroform extraction and subjected to long PCR (Expand Long Template PCR system, Boehringer Mannheim, Mannheim, Germany) to detect deletion of HTLV-I provirus. The primers of HTLV-I long terminal repeat (LTR) were 5′-LTR (5′-GTTCCACCCCTT-TCCCTTTCATTCACGACTGACTGC-3′) and 3′-LTR (5′-GGC-TCTAAGCCCCCGGGGGAT-3′) as described before.[37] Each 500 ng of genomic DNA was subjected to 10 cycles of denaturation (94°C, 10 sec), annealing (65°C, 30 sec) and extension (68°C, 8 min), and additional 20 cycles of denaturation (94°C, 10 sec), annealing (65°C, 30 sec) and extension (68°C, 8 min + 20 sec/cycle), then finalized by elongation of the product (68°C, 7 min). The PCR products were visualized by ethidium bromide staining after 0.6% agarose gel electrophoresis. The expected size of the amplified fragments with these LTR primers from a full-length HTLV-I provirus was 7.7 kbp. Long PCR products were partially sequenced on ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, Calif.) by using the Big Dye terminator and the primers pX4 (5′-GGGGAAGGAGGGGAGTCG-AGGGATAAGGAA-3′) or pX12 (5′-TTGCCCACCACCCTT-TTCCAGC-3′) in accordance with the manufacturer's instructions. Amino acid sequences at CTL epitopes, Tax 11-19 and Tax 301-309 restricted by HLA-A2 and A24, respectively, were then determined according to the nucleotide sequences.

Lymphocyte proliferation assay

For the mixed lymphocyte reaction (MLR), cryopreserved PBMC (2×10^5/well) from a healthy volunteer were co-cultured with formalin-fixed ATL cells with or without preculture *in vitro* (5×10^4/well) in 96-well U-bottom plates in triplicate at 37°C for 4 days. Cultures were pulsed with 37 kBq/well of [^{3}H]-thymidine ([^{3}H]-TdR) for an additional 16 hr to assess cell proliferation. Cells were harvested with a Micro 96 Harvester (Skatron, Lier, Norway) and [^{3}H]-TdR uptake into cells was measured in a microplate β counter (Micro Beta Plus; Wallac, Turku, Finland). Proliferation of HTLV-I-specific CTL (1×10^5/well) co-cultured with formalin-fixed ATL cells (5×10^4/well) for 2 days, and proliferation of rat spleen T cells (1×10^5/well) co-cultured with formalin-fixed various syngeneic rat cells (1×10^5/well) for 3 days were also similarly measured.

ELISA

Human and rat interferon-gamma (IFN-γ) production in 100 μL of culture supernatants was measured by Human IFN-γ ELISA kit (Endogen, Woburn, MA) and Rat IFN-γ ELISA kit (BioSource Inc., Camarillo, CA), respectively. Absorbances were measured at 450 nm using microplate reader (BioRad, Hercules, CA) and analyzed with Microplate Manager III software.

Inoculation of ATL cells in rats

Ten million formalin-fixed PBMC from ATL patients or uninfected healthy volunteers, with or without pre-culture *in vitro*, were subcutaneously administered to 4-week-old female F344n/+ rats twice with a 2-week interval. The rats were sacrificed at 1 month after second immunization. Spleen T cells from these rats were enriched through a nylon-wool column, and their IFN-γ production and proliferation against formalin-fixed syngeneic G14, G14-Tax or FPM1 cells were examined by IFN-γ ELISA and a [^{3}H]-TdR uptake assay, respectively, as described elsewhere.[38]

DNA-vaccination to rats

Plasmids containing wild-type *tax* cDNA controlled under the human β-actin promoter (pβMT-2 Tax) and its control plasmid pHβAPr.1-neo vector[39] were coated on Au particles and inoculated into rats by using Gene Gun as described previously.[14] Immunization was carried out 3 times with a 1-week interval. One week after final immunization, rats were sacrificed and spleen cells were collected.

Cytotoxicity assay

Spleen cells (5×10^6 cells) from immunized rats were used as effector cells after 7 days of co-culture with formalin-fixed G14-Tax (2×10^6 cells) in 24-well plate. Target cells (G14 or G14-Tax) were incubated with 370 kBq of [^{3}H]-TdR per 10^6 cells for 12 hours at 37°C, followed by extensive washing. These target cells (1×10^4/well) and effector cells (1×10^5/well) were plated in 96-well U-bottom plates at the effector/target ratio of 10. After 6 hr of incubation at 37°C, cells were harvested to glass filters and radioactivities remaining in the target cells were measured in a microplate β counter. The percentage of specific cell lysis was calculated as ([cpm without effector − cpm with effector]/cpm without effector) × 100.

Statistical analysis

Results are expressed as the mean ± SD. Differences between the 2 groups were analyzed for significance by Student's *t*-test. Differences among 3 groups were evaluated by Dunnett's *t*-test using SPSS Base 11.0J (SPSS Inc., Chicago, IL); *p*-values <0.05 were considered to be statistically significant.

Results

Detection of intracellular HTLV-I antigens in cell lines

Initially, to detect intracellular HTLV-I antigens by flow cytometry, the conditions of cell permeabilization and staining methods were determined using established cell lines. We used lysolecithin-paraformaldehyde, methanol and Triton-X to fix and permeabilize the cell membranes, and stained the cells with mAbs to HTLV-I p40 Tax (Lt-4), p24 Gag (NOR-1), and p19 and 28 Gag (GIN-7). The staining patterns under the optimal conditions are shown in Figure 1. HTLV-I-producing human T cell line MT-2, but not HTLV-I-negative Molt-4 cell line, was strongly stained with all of

these mAbs (Fig. 1*a,b*). Under the same conditions, an IL-2-dependent ILT-Hod cell line established previously from an ATL patient, exhibited 2 peaks, consisting of a large population weakly expressing HTLV-I antigens and a small population expressing substantial levels of the HTLV-I antigens (Fig. 1*c*). The levels of intracellular HTLV-I antigens, especially p40 Tax, fluctuated and were influenced by the culture conditions (data not shown).We also used saponin to permeabilize the cell membranes and compared the staining efficiency for detecting intracellular antigens with the methods using Triton-X. As shown in Figure 1*d*, saponin-treated ILT-Hod cells could also be stained with mAbs to HTLV-I but the detection levels were significantly lower than those in Triton-X-treated ILT-Hod cells. Thereafter, the permeabilization method using Triton-X was used primarily.

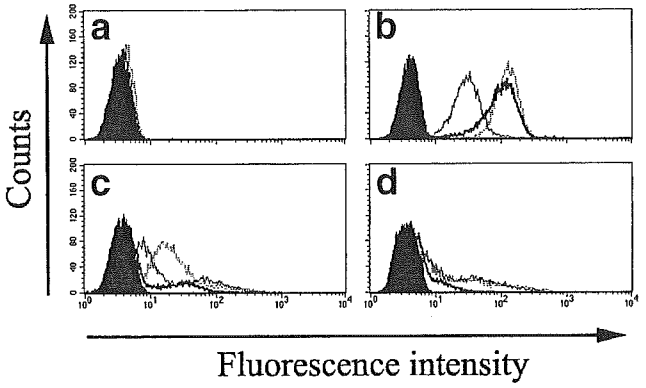


FIGURE 1 – Detection of intracellular HTLV-I antigens in cell lines. Molt-4 (*a*), MT-2 (*b*), and ILT-Hod (*c,d*) cells were permeabilized with Triton-X (*a–c*) or saponin (*d*) treatment (see Material and Methods), stained with control ascites (closed histogram), anti-p40 Tax mAb (Lt-4, solid lines), anti-p24 Gag mAb (NOR-1, thin lines) or anti-p19 and p28 Gag mAb (GIN-7, dashed lines) and subsequently with FITC-conjugated anti-mouse IgG + IgM. The cells were fixed with 1% formalin-PBS and analyzed using a flow cytometer.

Induction of HTLV-I antigens in PBMC from ATL patients

The clinical status of the ATL patients tested is summarized in Table I. Expression of HTLV-I antigens in ATL cells from 5 acute and 15 chronic ATL patients who had not received chemotherapy were investigated. Cryopreserved PBMC from ATL patients were permeabilized, and stained with mAbs against HTLV-I antigens, Lt-4, NOR-1 and GIN-7 immediately (Day 0) or after *in vitro* 1-day cultivation. When viral expression was detected in 1 day, cells were kept in culture for 3–9 days if available. Representative data of intracellular HTLV-I-expression in the PBMC from an acute ATL patient (Patient 85) is shown in Figure 2. Although HTLV-I antigens were not detectable in the PBMC of the ATL patients before culture, a large number of live cells strongly expressed HTLV-I antigens in a 1-day incubation. The HTLV-I-positive cell number increased with further incubation, whereas the live cell number decreased (data not shown). The intensity of p40 Tax in the positive population nearly reached the maximal level in 1 day, whereas the intensity of p24 or p19 and p28 Gag antigens was further enhanced in 3 days of incubation. Similar induction was observed in the PBMC of 3 (Patients 22, 85, 91) of 5 acute-type ATL patients tested, although the proportion of HTLV-I-

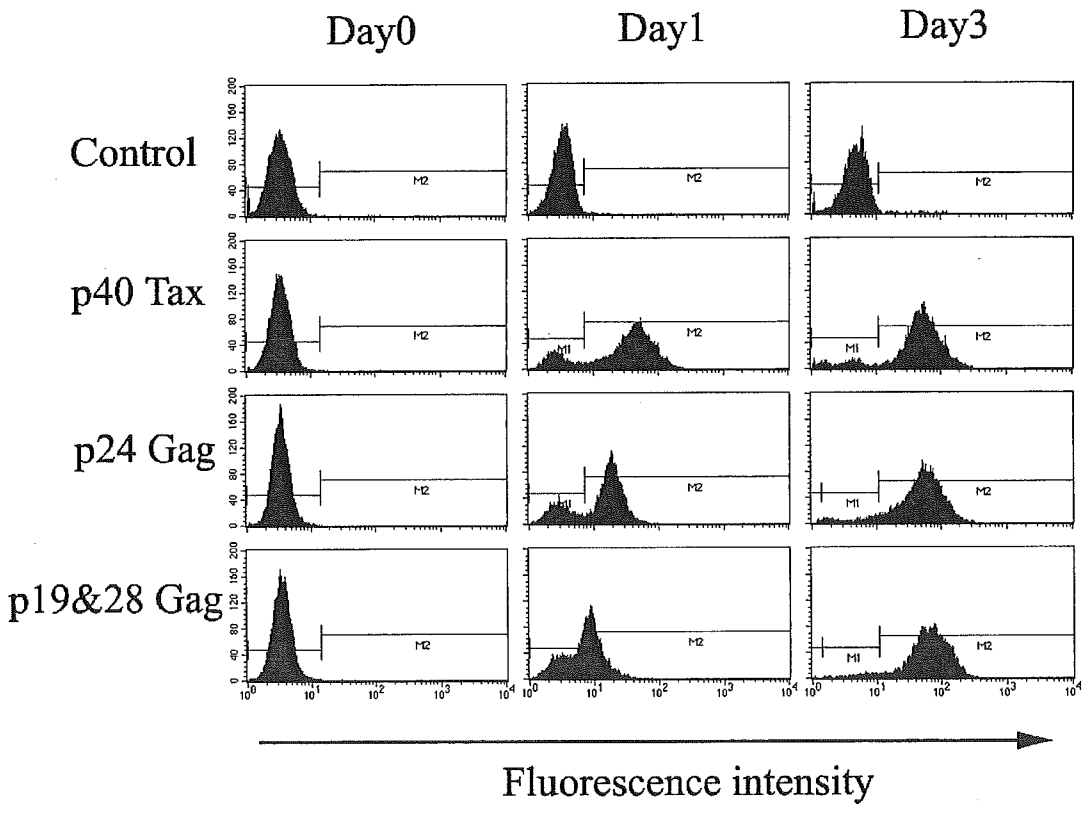


FIGURE 2 – Induction of HTLV-I antigens in PBMC of ATL patients after *in vitro* cultivation. Cryopreserved PBMC from Patient 85 (acute ATL) were incubated for the indicated periods in 10% FBS-RPMI, and permeabilized with Triton-X for intracellular staining with control ascites, Lt-4 (p40 Tax), NOR-1 (p24 Gag) or GIN-7 (p19 & p28 Gag) and subsequently with FITC-conjugated anti-mouse IgG + IgM. The M2 region shown in each histogram is regarded as positive.

expressing cells differed among individuals (Table II). In Patient 80 (acute ATL), only a small percentage of the cells expressed HTLV-I antigens after 9 days of incubation that probably arose from a minor population of the PBMC. The PBMC of Patient 1-vi who converted to acute-type ATL from chronic-type ATL (Patient 1-i) did not show any detectable levels of HTLV-I expression during 1 day of incubation.

In the chronic ATL patients, the results were more variable. In the PBMC of 3 (Patients 7, 79, 90) of 15 chronic ATL patients tested, more than 30% (range = 35.4–66.5%) of live cells expressed detectable levels of HTLV-I antigens in 1 day of incubation. In 3 chronic ATL patients (Patients 42, 54, 69), HTLV-I antigens were also induced but at a lower proportion (range = 6.8–11.3%) of the PBMC. No detectable levels of HTLV-I-expression were observed in the PBMC from the rest of the chronic ATL patients tested.

HTLV-I-induction was observed 3 of 5 acute-type and 6 of 15 chronic-type ATL patients, when HTLV-I-expression in >5% of the 1-day cultured PBMC was regarded as positive.

Conservation of representative CTL epitopes in ATL cells

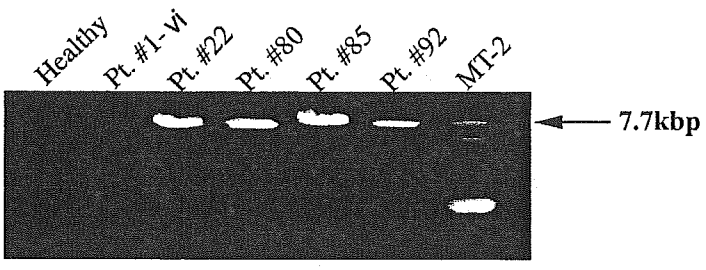
Because HTLV-I Tax is a major target antigen of HTLV-I-specific CTL,[16,17] we investigated whether ATL cells possessed mutations at the CTL epitopes in Tax. At first, the HTLV-I proviral genome integrated in the PBMC from 5 acute-type ATL patients was amplified by a long PCR method using LTR primers. As shown in Figure 3*a*, in 4 of 5 samples tested, comparable sizes of DNA fragments with a full-length provirus were amplified. No PCR product was obtained from the remaining case (Patient 1-vi). The DNA fragments amplified from the 3 patients were then examined for their nucleotide sequences at the regions corresponding to Tax 11–19 and Tax 301–309, representative CTL epitopes restricted by HLA-A2 and A24, respectively (Fig. 3*b*). Of the 4 acute-type ATL patients tested, 2 had HLA-A2, and all 4 had HLA-A24. The nucleotide sequences at Tax 11–19 were conserved in all patients regardless of the presence of HLA-A2. The sample from Patient 91 had a single mutation resulting in the substitution of serine (S) to asparagine (N) at the position 304. In the other 3 patients, nucleotide sequences at the Tax 301–309 region were identical to the prototype HTLV-I.[40]

TABLE II – INDUCTION OF HTLV-I ANTIGENS IN PBMC OF ATL PATIENTS FOLLOWING *IN VITRO* CULTIVATION

Patient	Type of ATL	Culture period (days)[1]	Percentage of positive cells for			
			Control antibody	Tax (Lt-4)	p24 (NOR-1)	p19&p28 (GIN-7)
#1-vi	Acute	0	0.1	0.0	0.0	0.1
		1	0.0	0.1	0.1	0.0
#22	Acute	0	0.0	0.0	0.0	0.0
		1	0.0	15.0	17.8	10.2
		3	0.0	26.4	56.4	38.1
#80	Acute	0	0.0	0.1	0.1	0.1
		1	0.0	0.5	0.3	0.2
		9	0.0	0.1	3.2	0.3
#85	Acute	0	0.1	0.0	0.0	0.0
		1	0.3	80.9	74.9	45.6
		3	0.6	91.4	93.9	97.3
#91	Acute	0	0.0	0.0	0.0	0.0
		1	0.2	31.4	25.8	22.5
#1-i	Chronic	0	0.0	0.0	0.0	ND[2]
		1	0.0	0.1	0.1	ND
#5	Chronic	0	0.0	0.0	0.0	0.0
		1	0.2	1.2	1.5	0.8
		9	0.1	0.9	7.0	6.7
#6	Chronic	0	0.2	0.1	0.1	ND
		2	0.1	0.2	0.1	ND
#7	Chronic	0	0.1	0.0	0.1	0.1
		1	0.2	52.3	66.5	62.1
#8	Chronic	0	0.1	0.1	0.1	0.0
		1	0.1	0.2	0.3	0.1
#20	Chronic	0	0.0	0.1	0.0	0.0
		1	0.3	0.5	0.4	0.4
#23	Chronic	0	0.0	0.0	0.1	0.0
		1	0.0	0.5	0.4	0.2
#29	Chronic	0	0.0	0.0	0.0	0.2
		1	0.1	0.3	0.4	0.1
#42	Chronic	0	0.0	0.0	0.0	0.0
		1	0.0	11.3	0.2	0.1
#54	Chronic	0	0.0	0.2	0.1	0.0
		1	0.1	9.7	10.5	5.4
#69	Chronic	0	0.0	0.3	0.2	0.1
		1	0.4	6.0	6.8	3.3
#79	Chronic	0	0.0	0.1	0.0	0.0
		1	0.0	63.4	59.2	33.2
#89	Chronic	0	0.0	0.0	0.0	0.1
		1	0.1	1.3	1.1	0.6
#90	Chronic	0	0.1	0.1	0.1	0.0
		1	0.1	35.4	32.0	15.9
#92	Chronic	0	0.0	0.0	0.0	0.0
		1	0.3	0.9	1.1	0.8

[1]Cryopreserved PBMC were thawed and expression of intracellular HTLV-I antigens were analyzed immediately (Day 0) or following incubation for the indicated days in 10% FBS RPMI. When the cells were cultured for longer than 3 days, 100 U/mL of IL- 2 was added to the culture medium to maintain cell viability.–[2]ND, not done.

a



b

Patients	HLA		Amino acid sequences	
	A2	A24	Tax11-19	Tax301-309
Pt. #1-vi	+	+	(not amplified)	(not amplified)
Pt. #22	+	+	LLFGYPVYV	SFHSLHLLF
Pt. #80	-	+	LLFGYPVYV	SFHSLHLLF
Pt. #85	-	+	LLFGYPVYV	SFHSLHLLF
Pt. #91	+	+	LLFGYPVYV	SFHN*LHLLF
Prototype HTLV-I			LLFGYPVYV	SFHSLHLLF

FIGURE 3 – Conservation of representative CTL epitopes in the proviruses from acute ATL cells. (*a*). Detection of full-length HTLV-I provirus in ATL cells. Genomic DNA (500 ng) extracted from PBMC of a healthy volunteer (*lane 1*), five acute ATL patients (*lane 2–6*), or MT-2 cells (*lane 7*) were amplified by a long PCR system with specific primers for 5′ and 3′ HTLV-I LTR. PCR products were visualized by ethidium bromide. The expected size of the fragments amplified from a full-length HTLV-I provirus was 7.7kbp in this system. (*b*) Nucleotide sequences of the long PCR products from acute ATL cells prepared in (*a*) were determined and the sequences at the regions corresponding to representative CTL epitopes, Tax 11-19 and Tax 301-309 restricted by HLA-A2 and A24, respectively, are shown as amino acid sequences. *Indicates the site of a single mutation (agt → aat) found.

Induction of co-stimulatory molecules in PBMC from ATL patients

It has been reported that HTLV-I-infected cell lines express a variety of surface molecules of activated T cells.[28] We investigated the expression of co-stimulatory molecules in ATL cells. It is known that typical ATL cells usually express CD4, CD25 and HLA-DR.[28] Besides these antigens, we assessed the expression of co-stimulatory molecules such as CD40, CD40L, CD80, CD86, OX40 and OX40L that participate in the interaction between antigen-presenting cells and T cells for efficient T cell-mediated immunity.[41] As shown in Figure 4, positive control ILT-Hod cells, an HTLV-I-infected T cell line established previously from an ATL patient, clearly expressed CD4, CD25, CD80, CD86, HLA-A, B, C and HLA-DR, and partially expressed OX40 and OX40L. The PBMC from an acute (Patient 85) and a chronic (Patient 79) ATL patient, that significantly expressed HTLV-I antigens after *in vitro* cultivation, were then analyzed before and after culture (Fig. 4). CD4, CD25 and HLA-A, B, C were detectable in uncultured PBMC from both patients. ATL cells from Patient 85 were double positive for CD4 and CD8. HLA-DR was detected in Patient 85 but not in Patient 79. In addition, small but detectable levels CD86 (14%) and OX40 (11%) were expressed in uncultured PBMC of Patient 85 and Patient 79, respectively (Day 0, closed histogram). After 1–3 days cultivation, expression of CD25, CD80, CD86 and OX40 was increased significantly. Similar induction of co-stimulatory molecules was also observed in other ATL cells with HTLV-I induction by culture (data not shown).

The results of two-color staining for HTLV-I Gag antigens and co-stimulatory molecules are shown in Figure 5. In 1-day cultured PBMC of Patient 85, the cells expressing intracellular HTLV-I Gag antigens partially expressed CD80 (23%) and CD86 (26%), and exclusively expressed OX40 (93%). This clearly indicated that HTLV-I and co-stimulatory molecules were co-expressed in ATL cells at the single cell level.

Among ATL cases without viral induction, the samples from Patient 1 expressed CD86 and OX40, and the sample from Patient 6 spontaneously expressed OX40 before culture (data not shown). The other samples tested did not express detectable levels of these co-stimulatory molecules. CD40 and CD40 ligand were not detectable in the PBMC of any ATL patients tested.

Augmentation of immunogenicity of ATL cells in vitro

We assessed the immunogenicity of short-term cultured ATL cells by *in vitro* MLR and HTLV-I-specific CTL assays. The results of MLR using allogeneic responder T cell and formalin-fixed PBMC from an acute (Patient 85) and a chronic (Patient 79) ATL patients were shown in Figure 6*a*. The levels of responder T cell proliferation were significantly enhanced upon mixing with 1-day or 3-day cultured ATL cells. Long-term cultured HTLV-I-infected T cell lines (ILT-85 and ILT-79) derived from these patients also induced significant levels of allogeneic MLR. It is of note that the ATL cells of Patient 79 expressed CD80 and OX40 but not HLA-DR (Fig. 4), suggesting that enhancement of MLR with ATL cells from this patient was not due merely to augmented HLA-DR. The levels of MLR against T cell-enriched fractions from 2 healthy volunteers were not markedly enhanced by preculture (Fig. 6*b*).

We assessed whether ATL cells could activate HTLV-I-specific CTL *in vitro* by mixing HLA-A24-restricted HTLV-I-specific $CD8^+$ CTL with formalin-fixed PBMC from HLA-A24-positive ATL patients (Patient 85 and Patient 1-vi) or a healthy volunteer. The results are shown in Table III. One- to three-day precultured, but not uncultured, PBMC of Patient 85 accelerated [^{3}H]-TdR-incorporation into the CTL, and induced marked levels of IFN-γ production in the CTL. In contrast, PBMC from a healthy volunteer or Patient 1-iv, in which HTLV-I antigens were not inducible, failed to enhance DNA synthesis or IFN-γ production in the CTL. Thus, short-term cultured ATL cells could be a specific stimulator as well as a target for HTLV-I-specific CTL in these *in vitro* assays.

In vivo *induction of HTLV-I-specific T cell responses by inoculating ATL cells in rats*

We investigated whether ATL cells could evoke HTLV-I-specific T cell immune responses *in vivo*. Twice with a 2-week interval, immunocompetent adult rats were subcutaneously inoculated with 10^7 cells/head of uncultured or 3 days-cultured PBMC from an acute ATL Patient 91 after formalin-treatment. Figure 7*a* shows the results of IFN-γ-production of spleen T cells from these rats 1 month after the last immunization. Surprisingly, in 2 of 2 rats inoculated with uncultured ATL cells, spleen T cells produced high levels of IFN-γ against stimulation with Tax-expressing syngeneic rat G14-Tax and HTLV-I-infected FPM1 cells but not with HTLV-I-negative G14

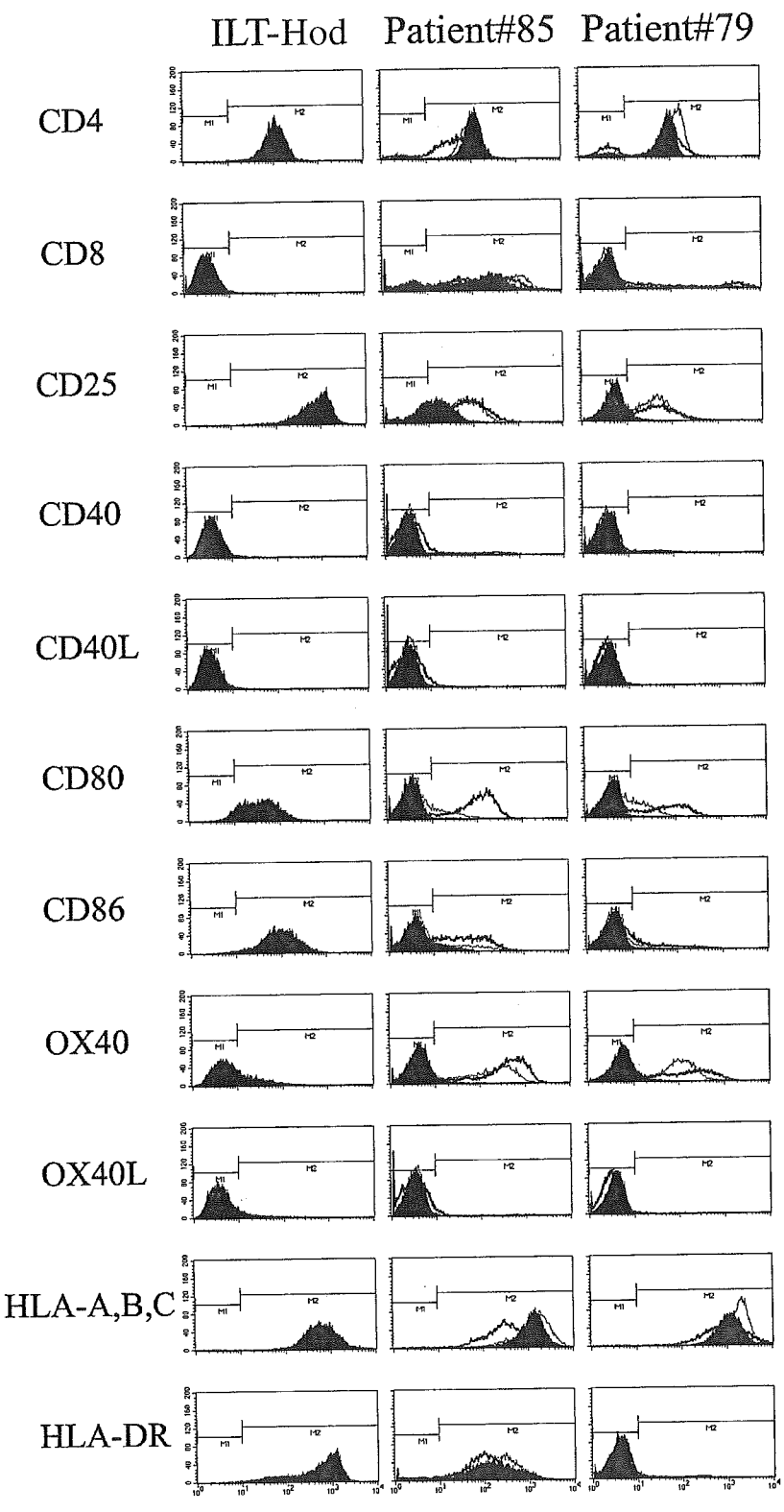


FIGURE 4 – Induction of co-stimulatory molecules in the PBMC of ATL patients after *in vitro* cultivation. Cryopreserved PBMC from an acute ATL Patient 85 and a chronic ATL Patient 79 were thawed and stained immediately (closed lines), or after incubation for 1 day (thin lines) or 3 days (solid lines) in 10% FBS-RPMI, FITC- or PE-conjugated anti-human mAbs (CD4, CD8, CD25, CD40, CD40L, CD80, CD86, OX40, OX40L, HLA-A, B, C, HLA-DR) as indicated. Cell surface antigen expression on ILT-Hod, an IL-2-dependent HTLV-I-infected cell line was analyzed by flow cytometry as a positive control. Live cells were gated and are shown as histograms. Each M1 region indicates where the cells stained with the isotype control mAb distributed (not shown). Accordingly, each remaining M2 region is regarded as positive.

cells (Fig. 7*a*; Rats 1 and 2). Similar or higher levels of HTLV-I-specific T cell responses were observed in the rats inoculated with 3-days cultured ATL cells from the same patient (Rats 3 and 4). T cells from control rats inoculated with PBMC from uninfected healthy human volunteers produced minimal levels of IFN-γ.

T cells from the rats inoculated with ATL cells also showed strong proliferative response against stimulation with G14-Tax and FPM1 cells but not with G14 cells (Fig. 7*b*). There was no significant difference between T cell response of the rats inoculated with uncultured and cultured ATL cells. CD4 positive cells became a dominant population in the spleen T cells from immunized rats after co-culture with formalin-fixed G14-Tax cells, whereas initially CD8 positive cells dominated before co-culture (Fig. 7*c*).

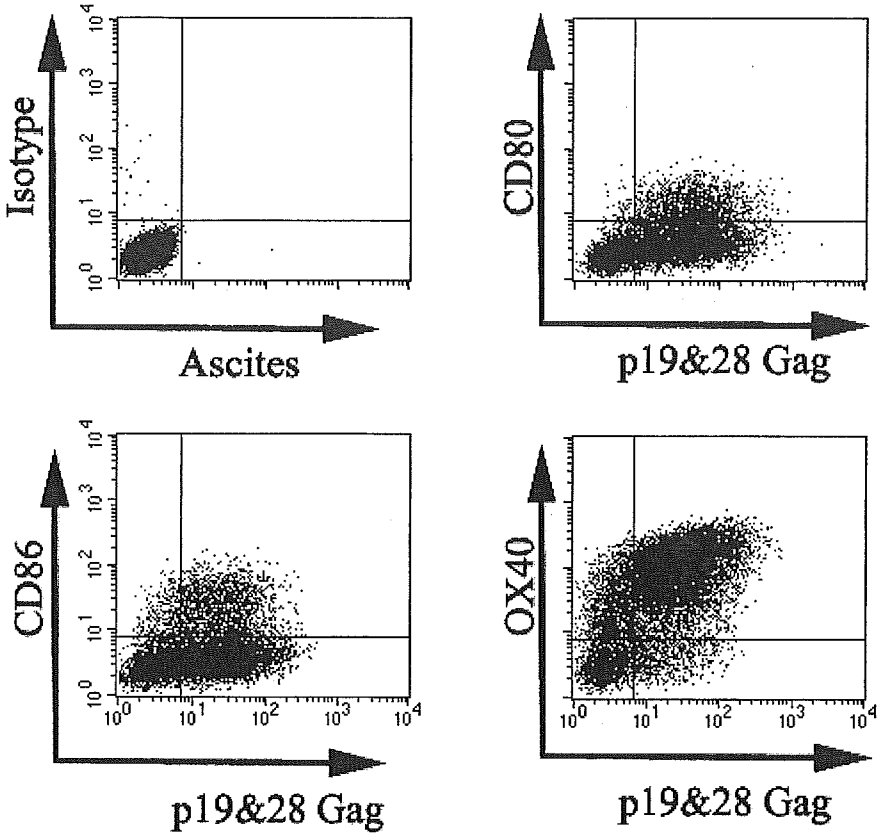


FIGURE 5 – Double induction of HTLV-1 and co-stimulatory molecules in the PBMC of ATL patients after *in vitro* cultivation. Cryopreserved PBMC from Patient 85 (acute ATL) were thawed and incubated for 1 day in 10% FBS-RPMI, and then subjected to a two-color analysis of cell surface antigens (CD80, CD86 or OX40) detected by FITC-conjugated mAbs and intracellular HTLV-I p19 and 28 Gag antigens detected by biotinylated GIN-7 mAb with Cy-Chrome-streptavidin after permeabilization with saponin (see Material and Methods). Live cells were gated and are shown as dot plots (FL-1/FL-3).

We tested cytotoxicity of the spleen cells from these rats inoculated with ATL cells after 7 days of *in vitro* co-culture with formalin-fixed G14 Tax cells. The results were shown in Figure 7*d*. The spleen cells from rats inoculated with ATL cells did not significantly kill Tax-expressing cells. In contrast, the positive control spleen cells from Tax-coding DNA-vaccinated rats showed strong cytotoxicity for G14-Tax but not for G14 cells.

These results suggest that not only precultured but also uncultured PBMC of ATL patients possessed sufficient amounts of antigens to evoke Tax-specific helper T cell response *in vivo*.

Discussion

We demonstrated that ATL cells retained the ability to express HTLV-I antigens including Tax in nearly 50% of the cases of ATL patients. Although the viral antigens became detectable in ATL cells by flow cytometric analysis only after short-term culture, the experiments with direct inoculation of the ATL cells to rats showed that uncultured ATL cells were also able to induce HTLV-I-specific T cell response *in vivo*.

It has been noted that HTLV-I expression is inducible in ATL cells from some, but not all, ATL patients.[26,28] In our present study, induction of HTLV-I Tax and Gag in ATL cells was observed in 3 of 5 acute ATL, 6 of 15 chronic ATL, and 9 of 19 ATL cases tested (Table II). In one case with chronic ATL (Patient 79), HTLV-I expression was induced in many more cells than the number of morphologically identified abnormal lymphocytes, indicating that many peripheral HTLV-I-infected cells could appear as normal lymphocytes. It is intriguing that HTLV-I-expression was induced more frequently in acute-type ATL cells than in chronic-type ATL cells, despite the fact that acute-type ATL is supposed to be at a more advanced stage than chronic-type ATL. During *in vivo* evolution of HTLV-I-infected cells toward ATL, modification of viral expression may not be an absolute requirement.

Nucleotide sequences at 2 representative CTL epitopes, Tax 11–19 and Tax 301–309 restricted by HLA-A2 and A24, respectively, were highly conserved in proviruses from 4 acute ATL patients tested (Fig. 3). We chose these epitopes because they were predominantly recognized by CTL in 2 ATL patients after hematopoietic stem cell transplantation,[29] and also because genomic frequencies of HLA-A2 and A24 in Japanese are 24.7% and 35.6%, respectively.[42] ATL cells retained their ability to express viral antigens in 3 of 4 patients with conserved epitopes. These observations suggest that CTL escape mutants may not be the main reason for ATL-development in these patients.

Direct inoculation of fresh ATL cells into naive rats efficiently induced HTLV-I-specific helper T cell response *in vivo* (Fig. 7), despite the apparent absence of HTLV-I antigens in these cells by flow cytometry. Because ATL cells were derived from human, the inoculated rats might potentially respond to xenogenic antigens. Because we used syngeneic rat target cells for the cytokine production assay to evaluate immune response in the rats, however, reactions against xenogenic antigens should not be picked up by this assay. In addition, because we treated uncultured ATL cells with formalin before inoculation to rats, immune response of the rats was not due to further induction of viral antigens in the ATL cells or secondary HTLV-I-infection *in vivo* either. Spleen T cells from the rats inoculated with ATL cells reacted with syngeneic HTLV-I-infected or Tax-expressing rat cells but not uninfected cells, indicating that these T cells recognized HTLV-I antigens including Tax or Tax-induced antigens.

The *in vivo* HTLV-I-antigenicity of fresh ATL cells described above contrasted with the observation that only precultured but not uncultured ATL cells activated HTLV-I-Tax-specific CTL line *in vitro* (Table 3). This may be partly explained by the different sensitivity of the responding T cells between *in vivo* and *in vitro* detection systems. The Tax-specific CTL line used in the *in vitro* assay has been established by a long-term culture, whereas the spleen T cell population of immunized rats would be more heter-

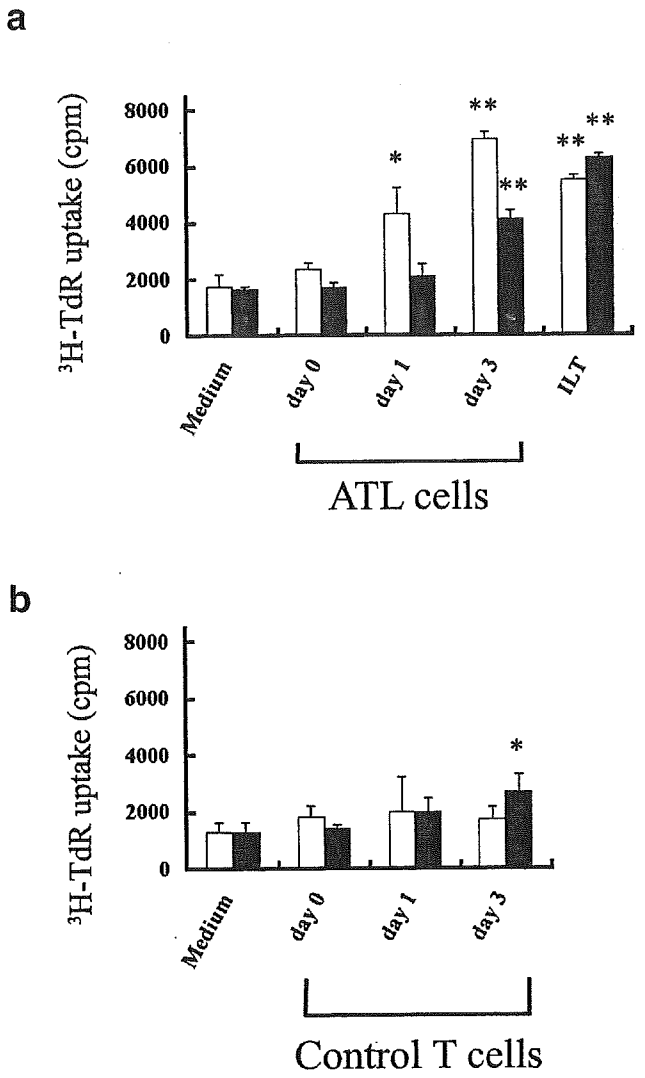


FIGURE 6 – Augmentation of antigenicity for allogeneic mixed lymphocyte reactions (MLR) in precultured PBMC from ATL patients. (*a*) PBMC from a healthy donor as responder cells were mixed with formalin-fixed PBMC from Patient 85 (acute ATL, open bar) and Patient 79 (chronic ATL, closed bar) that had been uncultured (Day 0) or precultured for the indicated periods (Day 1 or 3), or with formalin-fixed ILT cells derived from Patient 85 (open bar) and Patient 79 (closed bar). After 4 days of culture, [^{3}H]-TdR incorporated into the cells was measured. (*b*) Similar allogeneic MLR were carried out with responder PBMC from a healthy donor and formalin-fixed T cell-enriched PBMC fractions from two other healthy volunteers (open and closed bar) that had been precultured for the indicated periods. The results were expressed as the mean ± SD in counts per minutes (cpm) obtained from triplicate cultures. Differences between the 2 groups were analyzed for significance (*$p < 0.05$, **$p < 0.001$, compared to Day 0) by Student's *t*-test. Similar results were obtained in another set of independent experiments.

ogeneous. Antigens of formalin-fixed ATL cells inoculated to rats were presumably processed and presented mainly on MHC-II by professional antigen-presenting cells, which would favor CD4 positive helper T cell response *in vivo*. The amounts of antigen required for priming T cells *in vivo* might be smaller than those for activating the CTL line *in vitro*. In addition, the presence of co-stimulatory molecules on ATL cells might have been advantageous to induce T cell response *in vivo*.[41,43]

Short-term cultured ATL cells significantly expressed co-stimulatory molecules including CD80, CD86, and OX40 as well as HTLV-I antigens such as Tax and Gag at the single cell level. The levels of

TABLE III – PROLIFERATIVE RESPONSE AND IFN-γ PRODUCTION OF HTLV-I-SPECIFIC CTL IN RESPONSE TO ATL CELLS WITH OR WITHOUT *IN VITRO* PRECULTIVATION[1]

Stimulator PBMC		Responses of HTLV-I-specific CTL	
Subject	Preculture period (Day)	[^{3}H]-TdR uptake (cpm)	IFN-γ production (pg/mL)
Pt. #85	0	2,469 ± 246	Undetectable
	1	3,350 ± 423[2]	673 ± 45[3]
	3	4,498 ± 296[3]	534 ± 79[3]
Pt. #1-vi	0	2,493 ± 84	Undetectable
	1	2,470 ± 366	Undetectable
Healthy	0	2,737 ± 215	Undetectable
	1	2,554 ± 78	Undetectable
	3	2,748 ± 195	Undetectable
None	—	2,611 ± 115	Undetectable

[1]HLA-A24-restricted HTLV-I-specific CTL (1×10^5/well) were cocultured for 24 hr with formalin-fixed cryopreserved PBMC (5×10^4/well) from HLA-A24-positive ATL patients or healthy volunteers pre-incubated for the indicated periods (0, 1 or 3 days), and IFN-γ production in the culture supernatant and [^{3}H]TdR uptake for an additional 16 hr were measured. The results are expressed as the mean ± SD. Differences between the 2 groups were analyzed for significance.–[2]$p < 0.05$.–[3]$p < 0.001$, compared with Day 0 by Student's *t*-test. Similar results were obtained in another set of independent experiments.

Tax-expression in ATL cells reached the maximum in 1 day, whereas expression of Gag and co-stimulatory molecules increased with further incubation (3 days) (Fig. 4), suggesting that Tax was involved in the activation of the other molecules. A number of previous studies pointed to the potential transactivation of CD25,[44] OX40[45] and OX40L[46] by HTLV-I Tax. Induction of CD80 and CD86 in HTLV-I/II-infected cells has also been reported.[47] In our present study, spontaneous expression of OX40 and CD86 was sporadically observed in ATL cells before or without HTLV-I-induction. This indicates that Tax expression under detectable levels or some other mechanisms may be involved in activating co-stimulatory molecules in these ATL cells.

ATL may be categorized into at least 2 groups by the ability of HTLV-I-expression in their ATL cells. In our present study, HTLV-I expression was inducible in about half the ATL cases, and the other half showed irreversible viral silencing in their ATL cells. Although the irreversible silencing of HTLV-I may be due to various genomic changes in ATL cells,[24,25] HTLV-I expression is not completely silent in the other inducible type of viral suppression. This is supported by previous and recent reports that HTLV-I mRNA is detectable by RT-PCR in fresh ATL cells.[27,48] The inducible type of suppression is commonly seen in PBMC from HTLV-I-carriers and HAM/TSP patients.[22,49] Despite such suppression of viral expression *in vivo*, HTLV-I Tax-specific CTL are highly activated in HAM/TSP patients and some HTLV-I-carriers,[16,19] implying the presence of sufficient levels of antigen-presentation *in vivo* for priming and maintaining CTL. This is consistent with the observation in our present study that sub-detectable amounts of viral expression induced HTLV-I-specific T cell response *in vivo* but not fully activated Tax-specific CTL line *in vitro*. Such marginal levels of viral expression may partly explain how HTLV-I persists *in vivo* in the presence of HTLV-I-specific CTL. Nevertheless, active HTLV-I-specific CTL responses are associated with tumor-free state in human[16,21,29] and limited proviral loads in rats,[38] still suggesting contribution of HTLV-I-specific CTL to controlling expansion of HTLV-I-infected cells *in vivo*. It remains to be clarified where and when HTLV-I-specific CTL can affect infected cells *in vivo*.

Our results indicated that, in respect of the ability of viral expression, ATL has diversity even within the acute type ATL. In about half the ATL cases, ATL cells retained the ability of viral expression. Among these patients, fresh ATL cells from one case could induce Tax-specific helper T cell response *in vivo* despite their undetectable viral expression in *in vitro* assays. These imply that ATL cells may express low but sufficient levels of Tax or Tax-induced antigens to be recognized by T cells *in vivo*.

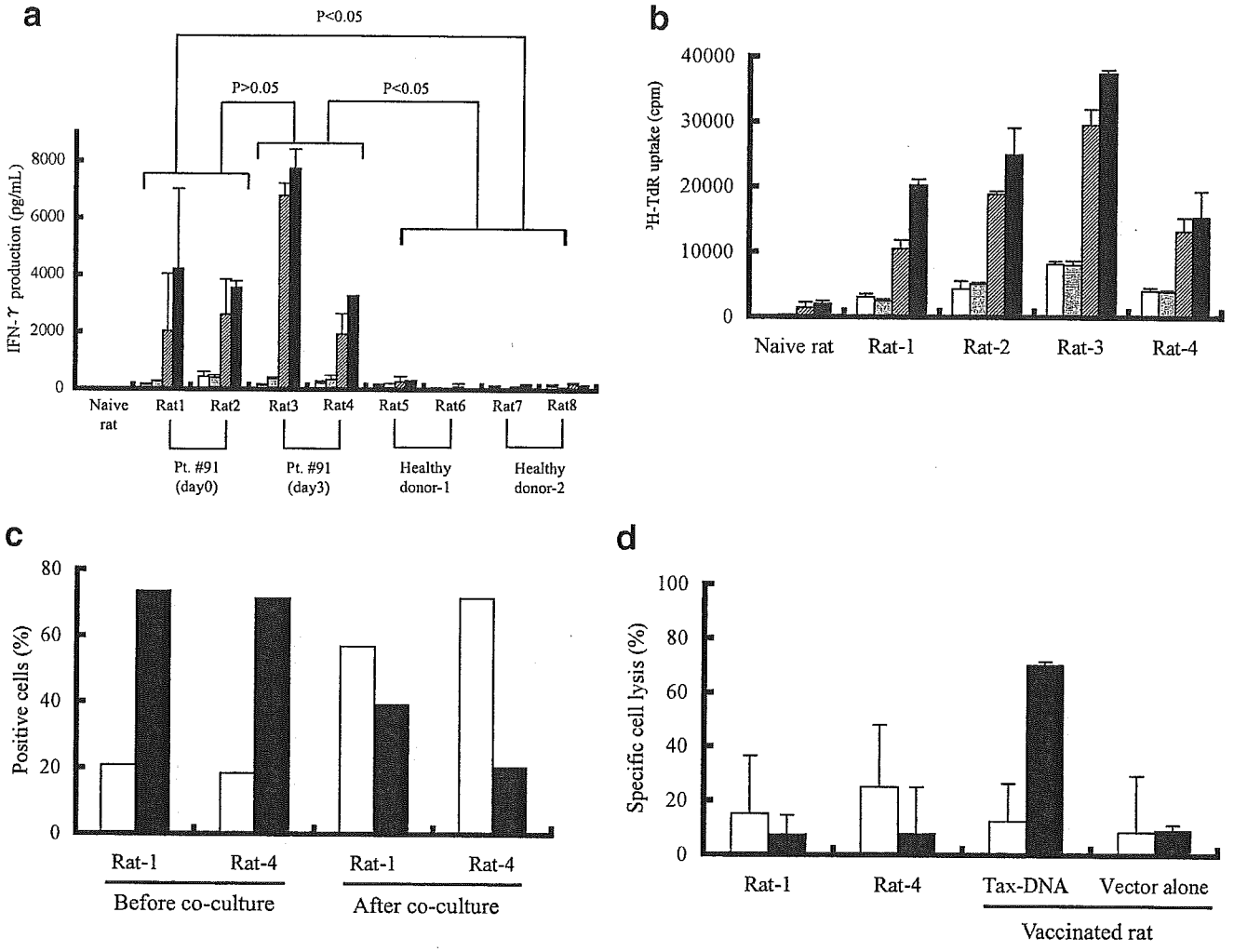


FIGURE 7 – *In vivo* induction of HTLV-I-specific T cell response by inoculation of fresh ATL cells in rats. Immunocompetent naive rats were subcutaneously immunized with either ten million of formalin-fixed ATL cells from Patient 91 that had been uncultured (Rats 1, 2) or 3 day-precultured (Rats 3, 4), or with the same number of formalin-fixed uncultured PBMC from 2 healthy donors (Rats 5–8), twice with a 2-week interval. One month after second immunization, spleen cells were collected. (*a*) IFN-γ production of spleen T cells from these rats were measured by ELISA after incubation without (open bar) or with formalin-fixed syngeneic G14 (gray bar), G14-Tax (hatched bar) or FPM1 (closed bar) cells for 6 days. Similar results were obtained also at the third day of incubation when half the medium was changed. (*b*) Proliferation of spleen T cells from immunized rats was monitored by a [^{3}H]-TdR-uptake assay after 3 days of incubation with various stimulator cells that were set up similarly to (*a*). (*c*) CD4 (open bar) or CD8 (closed bar) positive cell number in the spleen T cells from Rats 1 and 4 was evaluated by flow cytometry before and after 7 days of co-cultivation with formalin-fixed G14-Tax. Values indicate percent positive against viable cell number. (*d*) Cytotoxicities of spleen cells from Rats 1 and 4 were measured after 7 days of pre-culture with formalin-fixed G14-Tax. Similarly pre-cultured spleen cells from rats vaccinated with plasmids containing *tax* cDNA (Tax-DNA) or vector plasmids alone served as positive and negative controls, respectively. These effector cells were mixed with [^{3}H]-TdR-labeled G14 (open bar) and G14-Tax (closed bar) cells at the effector/target cell ratio of 10. After 6 hr incubation, cells were harvested and radioactivities remaining in the target cells were measured. Specific cell lysis were calculated (see Material and Methods) and expressed as the mean ± SD of triplicate cultures.

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Brief report

Allogeneic stem-cell transplantation with reduced conditioning intensity as a novel immunotherapy and antiviral therapy for adult T-cell leukemia/lymphoma

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Sixteen patients with adult T-cell leukemia/lymphoma (ATL) who were all over 50 years of age underwent allogeneic stem cell transplantation with reduced-conditioning intensity (RIST) from HLA-matched sibling donors after a conditioning regimen consisting of fludarabine (180 mg/m^2), busulfan (8 mg/kg), and rabbit antithymocyte globulin (5 mg/kg). The observed regimen-related toxicities and nonhematologic toxicities were all found to be acceptable. Disease relapse was the main cause of treatment failure. Three patients who had a relapse subsequently responded to a rapid discontinuation of the immunosuppressive agent and thereafter achieved another remission. After RIST, the human T-cell leukemia virus type 1 (HTLV-1) proviral load became undetectable in 8 patients. RIST is thus considered to be a feasible treatment for ATL. Our data also suggest the presence of a possible graft-versus-ATL effect; an anti-HTLV-1 activity was also found to be associated with this procedure. (Blood. 2005;105:4143-4145)

Introduction

Therapeutic trials to improve the dismal prognosis of adult T-cell leukemia/lymphoma (ATL) among elderly persons who are infected with human T-lymphotropic virus type 1 (HTLV-1) have so far been unsuccessful.[1-5] However, there have been a few encouraging reports on allogeneic stem cell transplantation (alloSCT) for selected populations of patients with ATL.[6-9] Although most of the patients who were treated successfully in these studies received grafts from HLA-identical siblings and the patients were younger than the average age for patients with ATL, the main cause of treatment failure after alloSCT remains transplant-related complications such as acute graft-versus-host disease (aGVHD). Recent advances have now allowed alloSCT to be extended to older patients through the use of reduced-intensity conditioning regimens.[10-12] We therefore conducted a phase 1 clinical trial of alloSCT with reduced-conditioning intensity (RIST) to clarify whether this newly developed procedure is feasible for ATL patients over 50 years of age.

Study design

The eligible patients ranged from 50 to 70 years of age and met the diagnostic criteria for ATL.[13] The patients were required to be in either complete remission (CR) or partial remission (PR) at the time of registration[5] and to have an HLA-identical sibling donor. All patients and donors gave their written informed consent to participate in this study, which was approved by the institutional review board of each participating institution.

The conditioning regimen consisted of fludarabine (180 mg/m^2), busulfan (8 mg/kg), and rabbit antithymocyte globulin (ATG; 5 mg/kg) as reported.[10] Granulocyte colony-stimulating factor-mobilized peripheral blood (PB) grafts from the donors were transplanted. To prevent GVHD, cyclosporine (CsA) was administered intravenously (3 mg/kg/d). The severity of GVHD was graded according to the consensus criteria.[14] The degrees of donor-recipient chimerism and HTLV-1 proviral DNA in PB mononuclear cells (MNCs) were quantified according to published methods.[15,16] The primary end points of this study were either engraftment, as judged by the achievement of complete donor chimerism before day 90, or the occurrence of early transplant-related mortality (TRM) before day 100 after RIST. We therefore registered 16 patients according to the Simon 2-step design.[17] The overall survival (OS) and event-free survival (EFS) were estimated by the Kaplan-Meier method. The log-rank test was used to compare the OS and EFS between the subgroups.

Results and discussion

Clinical results

The median ages of the patients and donors were 57 and 54 years, respectively. Because one patient (UPN11) received extra medication during the conditioning phase due to rapid disease progression, the patient was considered as evaluable only for engraftment. One

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Table 1. Patient characteristics and outcomes

UPN	Age, y/sex	ATL subtype	Donor HTLV-1 antibody	Complete chimerism, PB MNCs, > 90% of donor cells, d	GVHD		HTLV-1 proviral load		Outcome	Survival, d
					Acute	Chronic	Before RIST	After RIST, lowest level		
1	67/F	Acute	+	No	0	NE	292	68	LN relapse, d 47, DOD	135
2	61/F	Acute	−	14	IV	No	> 1000	2	LN relapse, d 47, CR after d/c CsA, died of aGVHD	173
3	62/F	Lymphoma	+	28	0	NE	30	43	LN relapse, d 14, DOD	43
4	62/M	Acute	+	14	1	Yes	> 1000	< 0.5	LN and skin relapse, d 28 and CR after d/c CsA	> 1214
5	51/M	Acute	−	42	0	No	709	223	LN and skin relapse, d 21, PR after d/c CsA, DOD	173
6	66/F	Acute	+	14	II	Yes	798	7	CR	> 1177
7	51/M	Acute	−	14	II	Yes	27	< 0.5	CR	> 1162
8	55/F	Lymphoma	+	20	0	No	331	67	LN relapse, d 74, DOD	201
9	53/M	Lymphoma	−	17	II	Yes	236	< 0.5	CR	> 1017
10	54/M	Lymphoma	−	17	II	Yes	440	< 0.5	LN relapse, d 171, CR after chemoradiotherapy	> 910
11	55/M	Acute	+	21	NE	NE	214	NE	NE	NE
12	66/F	Acute	−	14	0	Yes	> 1000	< 0.5	Died of cGVHD and infection	285
13	57/M	Acute	+	15	III	No	> 1000	2	LN and lung relapse, d 182, DOD	266
14	67/F	Lymphoma	−	15	III	No	582	< 0.5	LN relapse, d 62, DOD	219
15	54/M	Acute	+	28	III	NE	> 1000	< 0.5	Died of aGVHD and sepsis	71
16	56/M	Acute	−	14	IV	No	> 1000	< 0.5	Died of aGVHD	126

patient (UPN1) who developed an early relapse failed to achieve complete donor chimerism before day 90 (Table 1). Therefore, 15 of 16 patients were considered to demonstrate successful results for engraftment. Another patient (UPN15) developed early TRM on day 71 after RIST. As previously reported for this regimen, the regimen-related toxicities and hematologic toxicity were all acceptable. No grade 4 nonhematologic toxicity was observed.[10,18,19] Two patients developed fatal grade IV aGVHD while they were not receiving CsA because of an absence of aGVHD and an early disease relapse. Regarding major infectious complications, sepsis in 2 patients, a reactivation of cytomegalovirus in 13, and an Epstein-Barr virus-associated lymphoproliferative disorder in 2 were observed. Of the 12 patients who could be evaluated regarding the response to RIST, 9 exhibited CR at 30 days after RIST. Although the underlying mechanisms are unclear, the CR was considered most likely to be due to the chemotherapeutic effect, the graft-versus-ATL effect, or a combination of both. Disease relapse occurred in 9 patients. Interestingly, 3 patients who had a relapse subsequently achieved a second CR or PR after the rapid discontinuation of CsA. As of December 31, 2004, 5 patients are alive, and 10 had died of either ATL (6) or TRM (4). In all cases, TRM was considered to be related to GVHD (Table 1). The EFS and OS for the 15 patients at 2 years are 20.0% ± 10.3% and 33.3 ± 12.2%, respectively. The OS for patients who did and did not develop aGVHD was 50.0% ± 15.8% and 0%, respectively ($P = .06$).

Kinetics of the HTLV-1 proviral load after RIST

The HTLV-1 proviral load decreased to an undetectable level (< 0.5 copies) within 3 months after RIST in 8 patients, specifically, 6 of 8 patients who received grafts from HTLV-1 antibody-negative donors and 2 of 7 patients whose donors were virus carriers (Figure 1). Four of the 5 patients who survived more than 18 months presently continue to demonstrate an undetectable HTLV-1 proviral load. The other long-term survivor whose donor was a carrier (UPN6) showed a high HTLV-1 proviral load without any disease relapse beyond 18 months.

In this first prospective study of RIST for ATL, we clearly demonstrated that RIST from HLA-matched sibling donors is a feasible therapeutic procedure for patients over 50 years of age, as has been reported for other lymphoid malignancies.[20-22] However, the TRM of 27% was not negligible. Notably, 2 of 4 TRMs were related to grade IV aGVHD, and they were induced by a

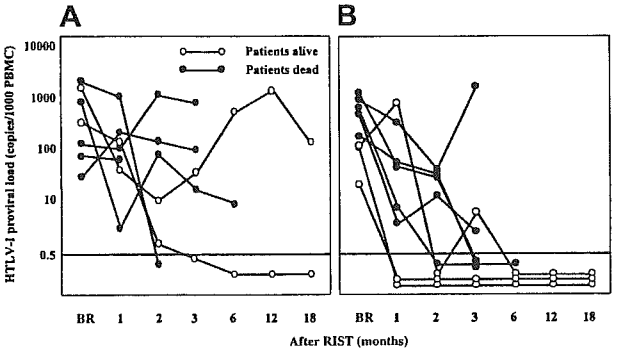

Figure 1. The kinetics of the HTLV-1 proviral load after RIST by different types of donors. Panel B indicates transplants from HTLV-1$^-$ donors; panel A shows results from HTLV-1$^+$ carrier donors. The HTLV-1 proviral load was expressed as copies per 1000 MNCs. A load of less than 0.5 copies/1000 MNCs was considered to be undetectable. ○ indicates patients still alive at end of study; ●, patients that died during study. BR indicates before RIST. The horizontal line at 0.5 indicates detection limit. PBMNC indicates peripheral blood mononuclear cell.