ATP受容体とパーキンソン病

現在のパーキンソン病治療薬

L-ドパは黒質の変性に伴う線条体のドパミン減少を補う基本的治療であるが，ドパミンの代謝は早く，また，ドパミン神経の変性に伴い蓄積できるシナプスも減少し，疾患の経過とともに効果の持続が短くなる。ドパミンアゴニスト（DA）はL-ドパの作用時間の短さを補うことができるが，嘔気，眠気，倦怠感が起こりやすく，また，最大効果はL-ドパで高い。アマンタジンはグルタミン酸受容体のNMDA受容体の拮抗作用によりPDの症状を改善させる。作用は強くないが，消化器症状や眠気などの副作用が少なく開始時には特にアドヒアランスがよく使いやすい。しかし，腎障害時には中毒を起こしやすく，PDの改善作用は十分ではない。抗コリン薬は著効する例も少なくないが，口渇や排尿障害，便秘などの副作用のため使いにくい。

アデノシンA_{2A}受容体拮抗薬は既存の治療薬とは作用部位が異なるために，これまでのPD治療薬では得られなかった新しい効果も期待される。

アデノシンの機能と受容体作用薬の作用

アデノシンはATPの代謝産物であるが，ATPが神経伝達物質とともに放出される時にシナプス間に放出されアデノシン受容体に作用する。また，放出されたATPからアデノシンに代謝されて受容体に作用する[1]（図1）。アデノシン受容体にはA_1，A_{2A}，A_{2B}，A_3のサブタイプがあり，中枢，末梢に分布している。

最もよく知られているアデノシン受容体拮抗薬はカフェインである。カフェインは非選択的にアデノシン受容体に作用し，覚醒作用，利尿作用，心機能への作用も認められる。アデノシン受容体拮抗薬のアミノフィリンは平滑筋の収縮に関与することから，喘息治療薬として用いられており，平滑筋を弛緩させ気管支を拡張させる。

アデノシンA_{2A}受容体は線条体のGABA/Enk細胞上に分布し，ドパミンD2受容体に対してはG proteinを介して抑制的な相互作用が想定されている[2]（図2）。細胞外のアデノシン濃度は20～300 nMであり，その濃度はA_{2A}受容体を刺激するのに十分であると考えられる[3]。これらの受容体は大脳基底核におけるindirect pathwayを制御しており，A_{2A}受容体拮抗薬はドパミンD2受容体に対するアデノシンの抑制を軽減させ，PDモデル動物の運動を改善させる[4]（図3,4）。また，モデル動物では線条体の細胞外アデノシンと代謝産物のイノシン濃度は増加しているとの報告もある[5]。なお，アデノシンA_{2A}受容体は淡蒼球にも分布し実験動物において淡蒼球への選択的な投与でもカタレプシー（無動）の改善が報告されており[6]，それぞれの受容体の役割については今後さらに研究が必要である。

コーヒーは非選択的なアデノシン受容体拮抗薬であるカフェインを主成分とするが，コーヒーの飲用者ではPDの発症が5分の1に減少するという疫学的な報告がなされている[7]。実験的にはアデノシンA_{2A}受容体拮抗薬は虚血やMPTPに対して保護的に作用する結果が報告されており[8][9]，アデノシン受容体と神経保護については今後の検討が待たれる。

アデノシン受容体拮抗薬とパーキンソン病の治療

アデノシン受容体拮抗薬のカフェインによる実験的な抗PD作用が報告されている。その作用は著明ではなくL-ドパとの併用により報告されている[10]。現在複数の選択的なアデノシン受容体拮抗薬が開発され，臨床研究が進んでおり，これまでの結果ではPDのL-ドパ治療におけるoff時間の短縮効果が確認されている[11]。我が国でも臨床試験が進められており，istradefyllineによるoff時間の短縮作用が確認された[12]。詳細な解析は今後進められるが，これまでの治療薬では得られなかった非運動症状への効果も期待される。

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BRIEF CLINICAL NOTES

レボドパ合剤で発症したParkinson病の突発性睡眠*

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Key Words: sleep attacks, levodopa, Parkinson disease, non-ergot dopamine agonist

非麦角系ドパミンアゴニストは突発性睡眠を起こすことがあり，添付文書上により警告され，服用時は機械の操作や自動車運転が禁止されている．しかし，非麦角系ドパミンアゴニスト以外の抗Parkinson病(PD)薬においても，突発性睡眠の報告は皆無ではない．われわれは，レボドパ合剤単剤での治療中に突発性睡眠発作が起こり，交通事故にあったPDの症例を経験した．注意喚起に有用な症例であると考えられるので報告する．

症　例

患者：62歳，女性．

主訴：バイク運転中の意識消失．

既往歴：特記事項はない．

家族歴：特記事項はない．

現病歴：2009年8月頃から四肢の筋強剛，動作緩慢が出現し，同年12月中旬に当科を受診した．PDと診断し，業務上運転が必要であったため，2010年3月中旬からレボドパ・カルビドパ合剤の内服を開始した．治療への反応は良く，同年4月下旬からは400/40mg/日(朝食後150/15mg，昼食後150/15mg，夕食後100/10mg)の内服により動作緩慢は改善し，オートバイで通勤していた．レボドパ・カルビドパ合剤を開始し，食後やテレビを見ているときなどに眠気を感じることはあったが，日常生活に支障はなかった．同年5月下旬，直線道路をバイクで運転中に突然意識を消失して転倒したため，当科外来を受診した．

来院時現症：体温36.2℃，血圧126/64mmHg，脈拍84/分・整．頭部に外傷の痕などはなかった．左下肢切創があった．神経学的には意識清明，脳神経領域に異常はなく，運動系では筋力は正常で筋萎縮などは認められなかったが，左側上下肢に軽度の筋固縮と動作緩慢を認めた．腱反射は正常で病的反射は陰性であった．感覚系に異常はみられなかった．歩行は軽度の前傾・小刻み歩行であったが，すくみや姿勢反射障害はみられなかった．起立性低血圧を含め自律神経障害も認められなかった．

検査所見：血液一般，血液生化学検査では異常は認められなかった．心電図は正常洞調律で不整は認められず，脳波でもてんかんを示唆す

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表1　ESS眠気の自己評価

0＝眠くなることはない，1＝ときどき眠くなる 2＝よく眠くなる，3＝だいたいいつも眠くなる	
1. 座って読書しているとき	0
2. テレビを見ているとき	0
3. 人の大勢いるところで座っているとき(会議場など)	1
4. 他の人が運転する車に休憩なしで1時間ほど乗っているとき	1
5. 午後，横になって休憩しているとき	1
6. 座って人と話をしているとき	0
7. 昼食後，静かに座っているとき	1
8. 車の運転中，渋滞などで2・3分停車しているとき	0
合計	4点

るような異常や，入眠期REMの所見なども認められなかった．頭部MRIでも正常であり，外傷を示唆する異常も認められなかった．

経過：頭頸部の外傷はなく，ヘルメットにも傷は認められなかった．頭部MRIも正常で，頭頸部の打撲を示唆するような異常はまったく認められなかった．眠気の評価も行ったが，Epworth Sleepness Scale(ESS)スコア[1](表1)は4点であり，過眠は認められなかった．突発性睡眠による転倒事故と考え，原因としてレボドパ合剤を疑い，本人の希望もあり内服を中止した(図1)．食後などの眠気は消失したが，PD症状が悪化するため，業務上運転が必要な朝は内服せず，昼・夕のみレボドパ合剤200/20mg(昼食後100/10mg，夕食後100/10mg)を内服するように調節したところ突発性睡眠は認めず，PD症状も軽減している．

考　察

突発性睡眠は非麦角系ドパミンアゴニストに特徴的な副作用と報告[2]され，非麦角系ドパミンアゴニストの親和性が高いdopamine D3受容体が関与すると考えられている[3]．しかし，その後の調査では麦角系でも発症しうることも知られており，多数例の調査ではレボドパ単独療法でも皆無ではない[4][5]．突発性睡眠発症の危険因子としては，男性であること，Hoehn & Yahrの重症度が低いことなどに加え，ESSスコアが10点以上で日中の眠気の存在があげられる[6]．しかし，突発性睡眠患者の10～18%においては，眠気の自覚がないとする報告[7]もあり，ESSスコア0点でも突発性睡眠を発症した症例の報告もみられる[5]．本例では日中の眠気の自覚はなかったが，突発性睡眠と考えられる意識消失が起こり事故につながっている．このことからレボドパ製剤のみで加療中で眠気の自覚がなくても，突発性睡眠に対する注意喚起は必要と考えられる．

まとめ

レボドパ合剤単剤でのParkinson病(PD)治療中にもかかわらず，バイク運転中に突然意識消失が起こり事故につながった突発性睡眠の62歳女性例を報告した．非麦角系ドパミンアゴニスト以外の抗PD薬で加療中で，日中の眠気を自覚していない症例であっても，突発性睡眠に対する注意は必要である．

本症例は第103回日本内科学会四国地方会で発表

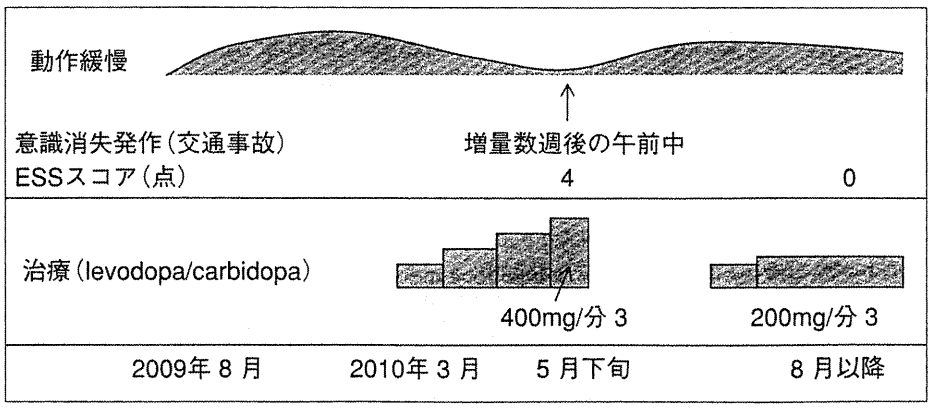

図1　経過図

した(2010年，愛媛)．

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<Abstract>

Sleep attack in Parkinson disease induced by levodopa treatment.

by

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We describe a 62-year-old female with Parkinson disease who experienced a sudden onset of sleep during treatment with levodopa/carbidopa. She fell asleep while riding her motorcycle on business in the morning. She fell off, injuring her arms. She had not previously experienced excessive somnolence. The sleep attack was successfully managed by taking levodopa/carbidopa after lunch and dinner and not in the morning. Patients with Parkinson disease receiving levodopa treatment should be informed of the possibility of sleep attacks.

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経口摂取不可時のParkinson病治療薬の検討

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〔要約〕 Parkinson病においてドパミン治療はactivities of daily living（ADL）を維持するために欠かすことのできないものであるが，抗Parkinson病薬はその殆どが内服薬であるために，内服ができない状況においては治療継続が困難となる．本邦においてはlevodopa注が唯一の非経口Parkinson病治療薬であるが，この薬の適切な使用方法についての情報は少ない．そこで我々は経口摂取不可時にlevodopa注の静脈内投与を行ったParkinson病症例においてlevodopa血中濃度を検討したところ，levodopa注を10mg/hrで投与すると血中濃度は約1μMとなることが明らかになった．

Levodopa注は単剤製剤で半減期が短いため，治療効果持続と副作用軽減の点から持続点滴する方法が適していると考える．「Levodopa注 10mg/hr＝Levodopa血中濃度1μM」をlevodopa注の投与量設定式として用いることを提案する．

（神経治療 28：677-680，2011）

Key Words：Parkinson's disease, levodopa, pharmacokinetics, continuous intravenous infusion of levodopa, plasma concentration of levodopa

はじめに

Parkinson病（Parkinson's disease：PD）は中脳黒質緻密層の神経細胞が変性脱落する疾患でドパミンが欠乏することでPD症状が出現する．PD症状はドパミンを補充することで改善がみられる．1960年にPD患者の脳でドパミンが減少しており，ドパミンの前駆物質であるDOPAを静注投与することで無動や振戦，筋強剛を一過性に軽減できることが報告された[1~3]．1960年代後半頃からlevodopaを大量に投与するとPD症状を劇的に改善できることが確認され，*dl*体に比べて*l*体が顆粒球減少などの副作用も少なく効果も大きいことが明らかにされた[4]．本邦では1971年にlevodopa内服薬が，次いで1972年にlevodopa注が承認発売された．1980年にlevodopa/ドパ脱炭酸酵素阻害薬配合薬（DCI）が登場してからはlevodopa単剤が用いられる機会は減った．その後多くの抗PD薬が開発されてきたが，注射製剤はlevodopa注だけである．

PDは中高年以上の年齢層に多いため，PD以外の病気を併発することもあり，内視鏡検査，超音波検査，造影剤を用いた画像検査，手術などが必要となることがある．また，肺炎や腸閉塞など全身状態が悪化して経口摂取ができなくなることもある．このような場合は当然内服薬では治療できない．

経口・経管投与ができないときのPD治療としては専らlevodopa注が用いられるが[5]，levodopa注を添付文書に沿った投与方法で用いても十分な治療効果が得られないことはしばしば経験される．

そこで我々は，経口摂取できないPD症例においてlevodopaの静脈内投与により加療を行い，同時にlevodopa血中濃度を測定し，経口薬の血中濃度と比較しながらlevodopa注の投与方法について検討した．

対　象

経口摂取不可時にlevodopa注の持続点滴を行ったPD患者5例（男性4例，女性1例，平均年齢71.2±3.1（mean±SD）歳，平均Hoehn & Yahr重症度分類4.2±0.2度，平均罹病期間10.8±3.2年，5例における総採血ポイント数29回）を対象とした（**Table 1**）．

方　法

Levodopa注を持続点滴中の任意の時間にヘパリン加スピッツを用いて採血を行った．遠心にて分離された血漿100μlに過塩素酸500μlを加え，その後再び遠心してタンパク質を除去，フィルターにて上清を濾過後high performance liquid chromatography（HPLC）にてlevodopa濃度を測定した．

結　果

測定されたlevodopa濃度を縦軸に，持続点滴levodopa注の投与量を横軸にプロットした（**Fig. 1**）．Levoopa注の持続点滴において投与量と血中濃度には有意な相関がみられた（$p<0.01$，$R^2=0.93$）．Levodopa注を10mg/hrで投与すると，血中濃度は1μMとなることが明らかになった．

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（2011年1月11日受付／2011年4月13日受理）

Table 1 Patients profile

Pt No/ Age/sex	Duration of PD (y)	H & Y stage	BW (kg)	Therapy (mg)
1/82/M	7	4	52	LD/CD 250
2/74/M	6	4	48	LD/CD 300, amantadine 100, zonisamide 25
3/67/M	4	4	65	LD/bens 400, selegiline 5, ropinirole 12, zonisamide 25
4/66/M	19	5	75	LD/CD 750, pramipexole 3.0, selegiline 7.5, amantadine 50
5/67/F	18	4	53	LD/CD 200, amantadine 200, zonisamide 100, trihexyphenidyl 1

LD/CD ; Levodopa/carbidopoa LD/bens ; Levodopa/benserazide
Dose of agents are shown in mg/day

症 例 1

症例1は82歳，男性．2001年74歳のときに右手の振戦が出現しPDと診断され内服治療を開始された．緩徐に症状は進行し2009年10月頃からは杖歩行となった．この頃，薬剤性の幻覚妄想状態が出現したため抗PD薬を減量し，levodopa/carbidopa 250mg/dayのみで加療していた．同年12月頻回の嘔吐が出現し当科受診し，腹部X線検査で腸閉塞と診断し緊急入院となった．

入院時の体温37℃，血圧171/84mmHg，脈拍71/分，腹部は膨満しており蠕動音聴取困難，打診にて鼓音あり，反跳痛は認めなかった．神経学的所見としては，意識レベルJCS 10，仮面様顔貌，小声で発語は聴取しづらい，右優位中等度筋強剛を認めた．四肢の麻痺はないが，無動は高度で巧緻運動は殆ど動作にならず，自力では寝返りも困難であった．血液検査でWBC 8,000/μl（Stab 8%，Seg 85%）の増多，CRP 1.86mg/dlと高値であった．一般生化学検査では肝・腎機能や電解質正常，CK 69IU/lと正常であった．腹部X線検査では著しく膨張した小腸ガス像を認め，胃泡は確認できなかった．腹部CT検査では狭窄起点は明らかでなく，麻痺性腸閉塞と診断した．腸閉塞に対して，絶飲食，補液，イレウス管の留置を行った．PDに対しては，levodopa注を20mg/hrで持続点滴を開始した．筋強剛は軽快したが，流涎が多く喀痰排出が困難であったため，levodopa注を30mg/hrまで増量した．増量後は嚥下動作もスムーズとなり，ベッド柵を持って自分で寝返りが可能になるなど，ベッド上のactivities of daily living（ADL）は維持できた．腸閉塞が軽快してからは通常の内服治療を開始し，levodopa注は中止した．Levodopa注持続点滴下におけるlevodopa血中濃度を測定したところ，20mg/hrでは2.66μM，2.88μMで，30mg/hrの投与時は3.59μM，3.95μMであった．嘔気・嘔吐，幻覚，興奮等の有害事象は認められなかった．

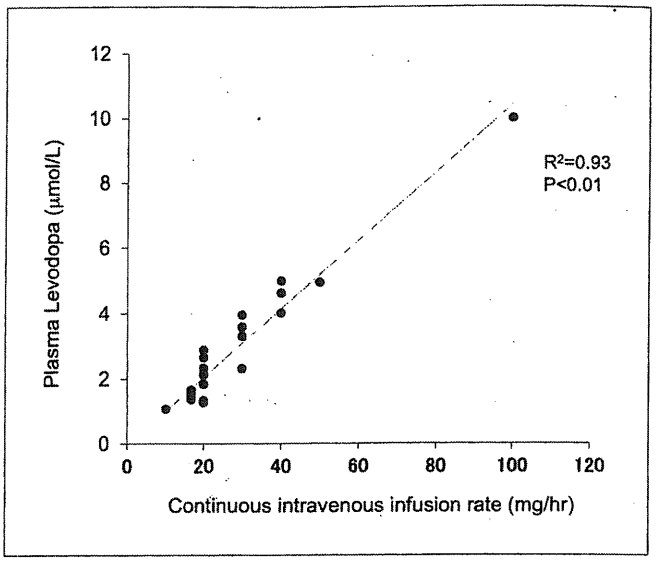

Fig. 1 Plasma concentrations of levodopa while injecting continuous intravenous levodopa in five cases
There was a significant correlation between administered dose and plasma concentration of levodopa.

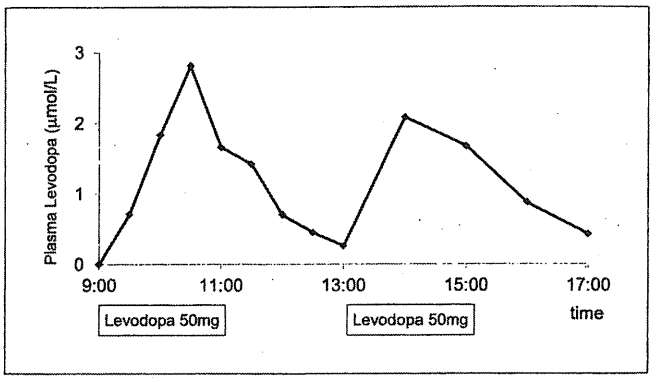

Fig. 2 Plasma concentration of levodopa while injecting intermittent intravenous levodopa

症 例 2

症例2は74歳，男性．2005年頃から左手の振戦が出現，PDと診断されL-dopa/DCI製剤の内服を開始した．2010年病状は徐々に進行し，歩行に介助が必要となった．同年8月上旬に旅行後，食欲低下，意識障害，発熱，四肢筋強剛の悪化を認め8月中旬近医入院．補液にて意識障害は改善したものの，誤嚥性肺炎を繰り返すため全身状態が悪化し当科転院となった．

神経学的には四肢高度な筋強剛と著明な廃用性筋萎縮を認め，Hoehn & Yahr 5度であった．経鼻胃管を留置して散剤にしたlevodopa/carbidopa 300mgで治療開始したが，治療効果が得られず，誤嚥性肺炎を繰り返すため経鼻胃管からの注入は中止とし，PD治療薬も注射製剤に切り替えた．最初にlevodopa注50mg点滴静注を1回2時間かけて，4時間毎に1日3回行った．その時の薬物動態を検討したところ，levodopaのCmaxはそれぞれ2.81μM，2.08μMと低値であり，静注終了後速やかに体内から消失した（**Fig. 2**）．この治療では十分なパーキンソニズムの改善が認められなかっ

た．次に，levodopa注の持続点滴を行った．20mg/hrの持続点滴では無効，30mg/hr以上で有効であったが，40mg/hrでは夜間譫妄の状態がみられた．これは減量後に回復した．

考　　察

今回我々は，腸閉塞を発症するなど全身状態悪化のために経口薬による治療が困難となったPD患者5例に対してlevodopa注の持続点滴を行い，その投与中のlevodopa血中濃度を測定した．

Levoopa注の持続点滴において投与量と血中濃度は相関がみられ，levodopa注を10mg/hrで投与すると血中濃度は約1μMとなることが明らかになった．この換算式に基づいて，約2～3μMを維持できるようにlevodopa注を20～30mg/hrの投与速度で持続点滴を行いながらPD症状のコントロールを行った．これは起床後から夜間まで1日10時間治療を行ったと仮定して，投与量としては200～300mgとなり，添付文書の記載量よりも数倍以上多い投薬量となるが，消化器症状や精神症状を含め特に副作用は認められなかった．

Levodopa注のインタビューフォームによると，「5例のPD患者に，levodopa注50mgを200mlの生理食塩液で希釈し，20分間で静注した時の血漿中levodopa濃度は静注開始後20分に最高値0.89mg/l（=4.5μM：著者注）となり，以後急速に減少した」とあり，用量・用法として1日levodopa注25～50mgを投与することと記載されている[5]．

Levodopa単剤静脈投与時の薬物動態に関する研究は限られており，その投与方法については参照できる文献やエビデンスに乏しい．

本邦のPD治療ガイドラインによると，イレウスを発症して経口・経管投与が困難な場合は，「Levodopa/DCI合剤100mgにつき50～100mgの割合で換算してLevodopaの静脈内投与が必要で，1日量を3回程度に分けて1回3時間程度かけて静脈内投与するのがよい」とある[6]．この方法に従うと，1日levodopa/DCI合剤300mgを内服している患者ではlevodopa注150～300mgの静脈内投与をする計算となり，投与量は我々の試算と同様となる．

我々の検討において，levodopa注50mgを間欠的に1回2時間かけて点滴静注した際のlevodopa血中濃度は，Cmax 3μMを下回っていた（Fig. 2）．一方PD患者4例においてlevodopa/DCI製剤1錠（100mg）を内服した時のlevodopa血中濃度は，個人差が大きいものの，最高血中濃度Cmaxは6.93±1.2μM（mean±SD）である[7]．これと間欠静注の血中濃度を比較してみると，levodopa注50mgではCmaxが半分以下と低値である（**Fig. 3**）．Levodopa注を間欠的に静脈内投与する場合，levodopa/DCI製剤1錠（100mg）の換算量としてlevodopa注100mg以上は必要であることが推測できる．適切な量を間欠的に静注することは，より経口薬に近い動態を形成できる利点があると思われる．

高齢の進行期PD患者に一時の代替治療としてlevodopa注を投与する場合には，安全に治療を行いながらADLを維持することが目的となるため，我々は持続注射での投与を選択し2～3μMのlevodopa血中濃度を維持した．これは治療量としては少ないが，ベッド上の生活が続くことによる拘縮，褥瘡，誤嚥性肺炎などの合併症予防を目的とする短期間の治療としては不足がなかった．

本研究はPD診療の中で経口摂取ができなくなった患者に対する限定的な少数例の検討であるが，実際の日常臨床の中で行われる治療に則して薬物動態を検討することは，持続点滴を行うにしても間欠的な静注を行うにしても，levodopa注治療に対して薬物動態をイメージしやすくなるため有用と考える．

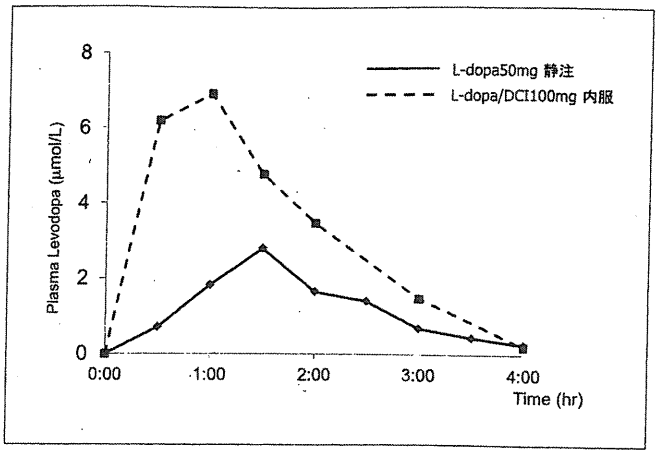

Fig. 3　Comparison of pharmacokinetics of levodopa between oral administration and intravenous injection of levodopa

インタビューフォームや添付文書の用量・用法ではPD症状を改善するのには不十分であることは明らかであり改訂が必要と思われる．また，PDにおける経口投与ができない場合の最適な治療方法について，個々の症例に応じた治療が選択できるように検討を重ねていく必要がある．

結　　論

Levodopa注は単剤製剤で半減期が短いため，治療効果持続と副作用軽減の点から持続点滴する方法は適している．Levodopa注の持続点滴においては，「Levodopa注 10mg/hr＝Levodopa血中濃度1μM」となることを，経口摂取不可時のlevodopa投与量設定として用いることを提案する．

本論文の要旨は第28回日本神経治療学会総会（2010年7月16日）において発表した．

［註］

（一般名）	（商品名）
levodopa注	ドパストン注
levodopa内服薬	ドパストン
levodopa/carbidopa	メネシット
levodopa/benserazide	イーシードパール
amantadine	シンメトレル
zonisamide	エクセグラン
selegiline	エフピー
ropinirole	レキップ
pramipexole	ビ・シフロール
trihexyphenidyl	アーテン

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Intravenous Levodopa Administration in Patients with Parkinson's Disease

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Almost all patients with Parkinson's disease (PD) are treated with oral medications. In case of suffering from gastrointestinal disorders, antiparkinsonian drugs require parenteral administration. Intravenous (IV) infusion of levodopa is the only available treatment for PD patients who are unable to take oral medications in Japan. An optimal IV infusion dosage of levodopa has not been clarified. Therefore we examined pharmacokinetics and effectiveness of IV infusion of levodopa. Five PD patients (four male and one female) treated with IV infusion of levodopa were intended in this study. Mean (SD) age, disease duration, and Hoehn and Yahr scale were 71.2 ± 3.1 years, 10.8 ± 3.2 years, and 4.2 ± 0.2, respectively. There was a significant linear correlation between given dose and plasma concentration of levodopa ($p < 0.01$, $R^2 = 0.93$). We found that plasma levodopa concentration retained approximately 1μmol/L when levodopa was kept on IV drips at a rate of 10mg per hour. Around 3μmol/L of levodopa plasma concentration is an adequate level for PD patients who require bed rest for a few days after surgery. This study contributes to determine optimal dosage of continuous IV infusion of levodopa.

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Target Epitopes of HTLV-1 Recognized by Class I MHC-Restricted Cytotoxic T Lymphocytes in Patients With Myelopathy and Spastic Paraparesis and Infected Patients With Autoimmune Disorders

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Human T-cell lymphotropic virus type I (HTLV-1) causes adult T-cell leukemia/lymphoma and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). The different patterns of clinical diseases are thought to be linked to immunogenetic host factors. A variety of autoimmune diseases, such as Sjögren's syndrome, have been reported in persons infected with HTLV-1, although the precise relationship between these disorders and HTLV-1 infection remains unknown. There is no report on the repertoire of HTLV-1-specific $CD8^+$ T-cells in HAM/TSP patients or carriers with autoimmune diseases, both characterized by an abnormal immune state. In this study, to characterize HTLV-1-specific $CD8^+$ T-cells in asymptomatic HTLV-1 carriers, HAM/TSP patients and carriers with autoimmune diseases, we examined the frequency and diversity of HTLV-1-specific $CD8^+$ T-cells using HTLV-1 tetramers. HTLV-1 Env-specific $CD8^+$ T-cells were significantly more frequent in HAM/TSP and carriers with autoimmune diseases compared with asymptomatic HTLV-1 carriers, while the frequency of HTLV-1 Tax-specific $CD8^+$ T-cells was not significantly different among them. $CD8^+$ cells binding to HTLV-1 Tax tetramers in carriers with autoimmune diseases were significantly reduced compared with HAM/TSP patients. This study demonstrates the importance of $CD8^+$ T-cells recognizing HTLV-1 Env-tetramers in HAM/TSP patients and carriers with autoimmune diseases, thereby suggesting that the diversity, frequency and repertoire of HTLV-1 Env-specific $CD8^+$ T-cell clones may be related to the hyperimmune response in HAM/TSP and carriers with autoimmune diseases, although different immunological mechanisms may mediate the hyperimmunity in these conditions. ***J. Med. Virol. 83:501–509, 2011.*** © 2011 Wiley-Liss, Inc.

KEY WORDS: HTLV-1; HAM/TSP; autoimmune diseases; MHC; CD8+ T cells

INTRODUCTION

Adult T-cell leukemia/lymphoma (ATL) [Poiesz et al., 1980; Hinuma et al., 1981; Tsukasaki et al., 2009] and human T-cell lymphotropic virus type I (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [Gessain et al., 1985; Osame et al., 1986; Casseb, 2009] are two of the most important diseases associated with long-term infection with HTLV-1, which has infected approximately 10–20 million people world-

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wide, particularly in Equatorial Africa, the Caribbean basin, South America, Melanesia, and southern Japan [Proietti et al., 2005]. The different patterns of clinical disease are thought to be linked to host immunogenetic factors. HTLV-1 is also associated with a variety of autoimmune disorders including T cell alveolitis, myopathy, uveitis, arthritis, and Sjögren's syndrome [Sugimoto et al., 1987; Vernant et al., 1988; Nishioka et al., 1989; Terada et al., 1994], although the precise relationship between these disorders and HTLV-1 infection remains unclear. Patients with systemic lupus erythematosus (SLE) and concomitant HTLV-1 infection, for example, seem to have a more indolent clinical course compared with patients with SLE who are not infected with HTLV-1 [Akimoto et al., 2007b].

HTLV-1 Tax-specific cytotoxic T lymphocytes (CTLs) play an important role in suppressing proliferation of HTLV-1-infected or transformed T cells in vitro [Jacobson et al., 1990; Bangham, 2008, 2009]. HTLV-1 Tax and envelope (Env) epitopes recognized by HLA class I molecules [Yashiki et al., 2001], and the association between HTLV-1 Tax-specific CTL frequency, as well as anti-HTLV-1 Tax antibody titers, with reduced HTLV-1 proviral load in asymptomatic HTLV-1 carriers have been described previously [Kozako et al., 2006, 2009c; Akimoto et al., 2007a]. In HAM/TSP, HTLV-1 specific $CD8^+$ CTLs target infected $CD4^+$ cells that have entered the central nervous system, resulting in direct and/or bystander injury [Ijichi et al., 1993]. These CTLs primarily target p40 Tax epitopes, although the less well-characterized *env* and *pol* encoded epitope targets have also been detected [Jacobson et al., 1991; Kannagi et al., 1991; Parker et al., 1992; Furukawa et al., 1994]. Like HAM/TSP, it has been demonstrated using vaccinia virus-HTLV-1 recombinants that $CD8^+$ CTLs from patients with Sjögren's syndrome show major histocompatibility (MHC) class I restricted cytotoxicity to target cells expressing various HTLV-1 proteins, primarily HTLV-1 Tax [Kannagi et al., 1991].

There appears to be no difference in the frequency of amino acid residues favoring MHC class I restricted binding when Env and Tax epitopes are compared, but among non-binding peptides, Env peptides seem more likely to have detrimental amino acid residues at the anchor sites [Pique et al., 1996]. Furthermore, because there seems to be no difference in the functional capacity of HTLV-1 virus-encoded envelope proteins originating from HAM/TSP patients compared with virus from ATL patients, host factors seem to play an important role in mediating immunopathogenesis [Pique et al., 1994]. Our current understanding of the immunological response to HTLV-1 envelope epitopes includes the broad immunogenicity of the surface protein Env175–199 that elicits helper T cell, cytotoxic T cell, as well as humoral responses [Baba et al., 1993]. Despite the apparent association between some autoimmune diseases and HTLV-1 infection, CTL responses targeting HTVL-1 Env epitopes have been less well characterized in individuals infected with HTLV-1 with autoimmune diseases.

MATERIALS AND METHODS

Subjects and PBMCs

The study sample consisted of 26 asymptomatic HTLV-1 carriers (range, 22- to 82-year-old; median, 60-year-old), 18 HAM/TSP patients (range, 34- to 73-year-old; median, 54-year-old), and 25 individuals with autoimmune disorders and HTLV-1 infection (range, 32- to 80-year-old; median, 60-year-old) from the Kagoshima University Hospital. The individuals with autoimmune disorders included eight with Sjögren's syndrome, seven with SLE, five with rheumatoid arthritis (RA), three with systemic sclerosis (SSc), and two with polymyositis (PM). Diagnoses of Sjögren's syndrome, SLE, and RA were made according to the revised Japan criteria for Sjögren's syndrome [Miyawaki, 2000], the revised criteria of the American College of Rheumatology (ACR) [Tan et al., 1982], and the 1987 ACR criteria [Silman, 1988], respectively. Anti-HTLV-1 antibody was measured by electrochemiluminescence immunoassay (ECLIA) (Picolumi®HTLV-I; Eisai, Tokyo, Japan), using beads coated with purified HTLV-1 antigen and synthetic Env peptides. This study protocol was in compliance with the Helsinki Declaration, and approved by the Medical Ethical Committee of Kagoshima University, and participants provided informed consent. Peripheral blood mononuclear cells (PBMCs) were separated from heparinized whole blood by centrifugation on Ficoll Hypaque (Amersham Biosciences, Uppsala, Sweden). For some experiments, cells were cryopreserved in liquid nitrogen until assayed [Kozako et al., 2006, 2009b,c].

HLA Typing of PBMCs

Based on prior reports, HLA type analysis revealed that 88% of people infected with HTLV-1 were HLA-A*02 or HLA-A*24 [Kozako et al., 2006], consistent with other studies of HLA allele types in the population of Southern Kyushu, Japan [Sonoda et al., 2000]. PBMC samples were screened initially by serological staining with monoclonal antibodies (mAbs) for HLA-A*02 supertype (clone BB7.2) and HLA-A*24 supertype (clone 17A10; Medical and Biological Laboratories, Nagoya, Japan), followed by secondary staining with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Immunotech, Praka, Czech) and subjected to flow cytometry on a FACScan (BD Biosciences, San Jose, CA). Individuals with neither HLA-A*02 nor HLA-A*24 were excluded from this study. HLA allele types of asymptomatic HTLV-1 carriers were also confirmed by the Luminex method using DNA isolated from cryopreserved PBMCs, as has been previously described (G&G Science, Fukushima, Japan) [Itoh et al., 2005].

Tetramer Assay for HTLV-1 Tax/Env-Specific CD8+ T Cells

Sixteen distinct phycoerythrin (PE)-conjugated HLA-A*0201 and HLA-A*2402 tetramers for HTLV-1 Tax and Env peptides (Medical and Biological Laboratories)

based on known HTLV-1 Tax and Env CTL epitope mapping data were used in this study [Yashiki et al., 2001] (Table II). HLA tetramers were produced as previously described [Baenziger et al., 1986; Altman et al., 1996; Kozako et al., 2006, 2009c]. Aliquots of 1×10^6 PBMCs were incubated with each of 16 distinct HTLV-1 Tax or Env peptides, followed by staining with FITC-conjugated mouse anti-human CD8 mAbs (Beckman Coulter, Fullerton, CA) and peridin chlorophyll a protein (PerCP)-conjugated anti-CD45 (BD Biosciences) according to the manufacturer's instructions. $CD45^+$ lymphocytes were applied to a FACScan [Kuzushima et al., 2001] and 1×10^5 events analyzed with FlowJo software (Tree Star, San Carlos, CA) [Betts et al., 2004]. Human immunodeficiency virus (HIV)/HLA tetramers (Medical and Biological Laboratories) were also stained as negative controls. Based on the negative controls, a cut-off point of 0.1% for HTLV-1/HLA tetramer positivity in $CD8^+CD45^+$ T lymphocytes was used, as previously described [Akimoto et al., 2007a; Kozako et al., 2006].

Statistical Analysis

The proportion of people with HTLV-1-specific $CD8^+$ T cell positivity for respective subject groups was compared with a χ^2 test or Fisher's exact test as appropriate. The percentage of tetramer positive cells was compared using the Mann–Whitney *U*-test. SPSS for Windows (version 14.0J; SPSS, Inc., Chicago, IL) was used for statistical analyses, and $P < 0.05$ was considered statistically significant.

RESULTS

Adopting HLA-A*0201 Tetramers for Carriers Possessing HLA-A*0201 and HLA-A*0206

Consistent with prior studies in the Southern Kyushu population [Sonoda et al., 2000], most of the asymptomatic HTLV-1 carriers with the HLA-A*02 haplotype were either HLA-A*0201 or HLA-A*0206, and nearly all HLA-A*24 subjects were HLA-A*2402. Of 26 asymptomatic HTLV-1 carriers, six were HLA-A*0201, five were HLA-A*0206, 18 were HLA-A*2402, and three were heterozygous for HLA-A*0201 and HLA-A*2402. Because existing HTLV-1/HLA tetramers were developed for carriers possessing HLA-A*0201 or HLA-A*2402, but not for HLA-A*0206, HLA-A*0201 tetramers were adopted for subjects of the HLA-A*0206 haplotype. In comparing asymptomatic HTLV-1 carriers with HLA-A*0201 and HLA-A*0206 haplotypes, there was no significant difference in frequency of Tax-specific $CD8^+$ T cells (27% and 16%, respectively; $P = 0.52$), or the proportion of individuals with detectable $CD8^+$ T cells for all HTLV-1 tetramers tested (Table I). Furthermore, the CMV p65/HLA-A*0201 tetramer was recognized by $CD8^+$ T cells from individuals with HLA-A*0201 ($n = 6$) and HLA-A*0206 ($n = 4$) haplotypes. These normally HLA-A*0201-restricted tetramers were thus also considered to be reliable in selecting $CD8^+$ T cells in HLA-A*0206 individuals. The lower limit of this assay for detecting tetramer specific $CD8^+$ T cells was 0.1%, defined using HIV-1 tetramers as negative control, and consistent with previous reports [Kozako et al., 2006, 2009a,b,c]. The percentage of HTLV-1 tetramer positive cells in the $CD8^+CD45^+$ T cell subset ranged from 0% to 1.28% (Fig. 1).

Env-Derived Epitopes are Recognized More Frequently by Patients With Autoimmune Diseases

The proportion of individuals with detectable $CD8^+$ T cells binding the various envelope epitope tetramers tested was significantly higher in HAM/TSP patients and carriers with autoimmune diseases (15% and 25%, respectively), compared with asymptomatic HTLV-1 carriers (1%, $P < 0.001$, Table II). There was no statistically significant difference in the proportion of individuals with detectable $CD8^+$ T cells specific for the different HTLV-1 Tax epitope tetramers. Individuals with autoimmune diseases had detectable $CD8^+$ T cells that primarily recognized envelope-derived epitopes to a greater extent than Tax-derived epitope tetramers. A

TABLE I. Frequency of HTLV-1-Specific CTL Positivity in Asymptomatic HTLV-1 Carriers Possessing HLA-A*0201 and HLA-A*0206

Tetramers	HLA-A*0201	HLA-A*0206
T11	100% (6/6)[a]	60% (3/5)
T123	17% (1/6)	0% (0/5)
T155	0% (0/6)	0% (0/5)
T178	0% (0/6)	20% (1/5)
T307	17% (1/6)	0% (0/5)
E175	0% (0/6)	0% (0/5)
E239	0% (0/6)	0% (0/5)
E442	0% (0/6)	0% (0/5)
HTLV-1 Tax CTL positives	27% (8/30)	16% (4/25)*
HTLV-1 Env CTL positives	0% (0/18)	0% (0/15)
Total HTLV-1-specific CTL positives	17% (8/48)	10% (4/40)**
CMV pp65-specific CTL positives	100% (6/6)	100% (4/4)

[a]Epitopes detected by HTLV-1/HLA tetramers/number of tetramers tested.
*$P = 0.52$.
**$P = 0.55$.

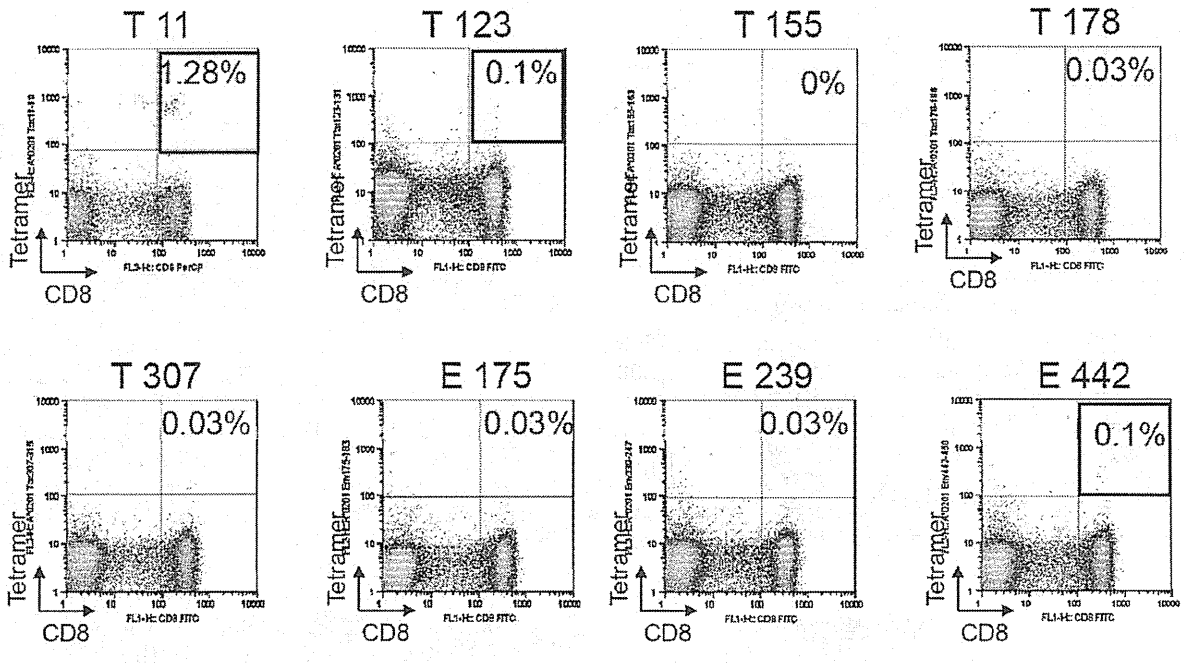

Fig. 1. Variety of HTLV-1-specific $CD8^+$ T cells in fresh PBMCs. $Tetramer^+CD8^+$ T cells were estimated among the $CD45^+$ T lymphocyte population. Fresh PBMCs isolated from carriers with autoimmune diseases were stained with eight distinct HTLV-1/HLA-A*0201-tetramers (T11, T123, T155, T178, T307, E175, E239, and E442). Numbers in the upper right quadrants represent the percentages of $tetramer^+CD8^+$ T cells among $CD8^+CD45^+$ T lymphocytes.

higher proportion of HTLV-1 infected individuals with Sjögren's syndrome, SLE, or SSc had detectable levels of envelope tetramer specific $CD8^+$ T cells (30%, 29%, and 33%, respectively), compared with asymptomatic carriers (1.1%, $P < 0.001$). In contrast, the proportion of individuals with detectable Tax-specific $CD8^+$ T cells was not significantly different among the subject groups, except for individuals with Sjörgren's syndrome, who were more likely than asymptomatic carriers to have detectable Tax-specific $CD8^+$ T cells (34% vs. 22%, respectively). In fact, individuals with Sjörgren's syndrome were more likely to have detectable overall tetramer (Tax- and Env-) specific $CD8^+$ T cells compared with asymptomatic carriers (34% vs. 14%, respectively; $P < 0.001$). There were no significant differences observed in the proportion of individuals with detectable tetramer specific $CD8^+$ T cells when individuals with rheumatoid arthritis or polymyositis were compared with asymptomatic carriers for Tax-specific (24%, 40%, and 22%, respectively) or Env-specific $CD8^+$ T cells (6.7%, 17%, and 1.1%, respectively). Table III summarizes the proportion of individuals with detectable $CD8^+$ T cells binding the various epitope tetramers tested. There was no significant difference in the proportion of individuals with $CD8^+$ T cells binding Tax-derived epitope tetramers, but a higher proportion of HAM/TSP patients (50%) and carriers with autoimmune diseases (64%) had Env-tetramer specific $CD8^+$ T cells overall, when compared with asymptomatic HTLV-1 carriers (3.8%, $P < 0.001$).

$CD8^+$ T Cells From Asymptomatic HTLV-1 Carriers Exhibit a Narrower HTLV-1 Epitope Repertoire Than HAM/TSP Patients and Carriers With Autoimmune Diseases

Fifty-nine individuals were assessed using eight distinct tetramers corresponding to the immunodominant HLA-A*02 or HLA-A*24 restricted epitopes. In the case of individuals with both HLA alleles (three asymptomatic HTLV-1 carriers, seven HAM/TSP patients, and three HTLV-1 carriers with autoimmune diseases), epitope-specific $CD8^+$ T-cells were analyzed using eight tetramers for each HLA type. The array of HTLV-1 epitopes recognized by $CD8^+$ T cells varied, not only by HLA/HTLV-1 peptide, but also according to clinical diagnosis (Table II). Among asymptomatic HTLV-1 carriers, $CD8^+$ T cells predominantly recognized the HLA-A*0201-restricted Tax11–19 and the HLA-A*2402-restricted Tax301–309 tetramers. None of the asymptomatic HTLV-1 carriers in this study had detectable $CD8^+$ T cells binding T155, T289, or T311 tetramers (Table II), whereas these tetramers were recognized by HAM/TSP patients and HTLV-1 infected carriers with autoimmune diseases. Envelope epitopes were rarely recognized by $CD8^+$ T cells from asymptomatic carriers, while HAM/TSP patients and carriers with autoimmune diseases consistently showed detectable levels of Env-specific $CD8^+$ T cells binding HLA-A*0201-restricted Env175–183, Env239–246, Env442–450; and HLA-A*2402-restricted Env11–19,

TABLE II. Diversity of HTLV-1 Epitopes Recognized by CTL m Asympiomatic HTLV-1 Carriers, HAM/TSP Patients, and Earners With Autoimmune Diseases

Tetramers	HLA allele	HTLV-l peptide	Asymptomatic HTLV-1 carriers	HAM TSP	Carriers with autoimmune diseases
T11	A*0201	Taxll–19	73% (8/11)[a]	90% (9/10)	55% (6/11)
T123	A*0201	Taxl23–131	9% (1/11)	20% (2/10)	45% (5/11)
T155	A*0201	Taxl55–163	0% (0/11)	10% (1/10)	0% (0/11)
T178	A*0201	Taxl78–186	9% (1/11)	20% (2/10)	18% (2/11)
T3O7	A*0201	Tax307–315	9% (1/11)	0% (0/10)	0% (0/11)
E175	A*0201	Envl75–183	0% (0/11)	0% (0/10)	9% (1/11)
E239	A*0201	Env239–247	0% (0/11)	10% (1/11)	18% (2/11)
E442	A*0201	Env442–450	0% (0/12)	0% (0/12)	18% (2/12)
T12	A*2402	Taxl2–20	11% (2/13)	13% (2/13)	24% (4/13)
T187	A*2402	Taxl87–195	11% (2/18)	7% (1/15)	18% (3/17)
T289	A*2402	Tax289–297	0% (0/18)	13% (2/15)	18% (3/17)
T301	A*2402	Tax301–309	94% (17/18)	87% (13/15)	76% (13/17)
T311	A*2402	Tax311–319	0% (0/16)	7% (1/16)	6% (1/16)
E11	A*2402	Euvll–19	6% (1/17)	40% (6/17)***	47% (8/17)***
E21	A*2402	Env21–29	0% (0/18)	13% (2/18)	35% (6/18)***
B153	A*2402	Envl 53–161	0% (0/18)	13% (2/I?)	24% (4/17)
Tax CTL positives			22% (32/145)	26% (33/125)	26% (37/140)
Env CTL positives			1% (1/87)	15% (11/75)*	27% (23/84)*
Total CTL positives			14% (33/232)	22% (44/200)***	27% (60/224)**

Tetramers	Sioaren's syndrome	SLE	RA	Sarcoidosis	PM	SSc
T11	100% (3/3)	25% (1/4)	33% (1/3)	100% (1/1)	100% (1/1)	NT
T123	67% (2/3)	50% (2/4)	33% (1/3)	0% (0/1)	0% (0/1)	NT
T155	0% (0/3)	0% (0/4)	0% (0/3)	0% (0/1)	0% (0/1)	NT
T178	0% (0/3)	25% (1/4)	33% (1/3)	0% (0/1)	0% (0/1)	NT
T307	0% (0/3)	0% (0/4)	0% (0/3)	0% (0/1)	0% (0/1)	NT
E175	33% (1/3)	0% (0/4)	0% (0/3)	0% (0/1)	0% (0/1)	NT
E239	33% (1/3)	25% (1/4)	0% (0/3)	0% (0/1)	0% (0/1)	NT
E442	33% (1/3)	25% (1/4)	0% (0/3)	0% (0/1)	0% (0/)	NT
TI2	29% (2/7)	0% (0/4)	0% (0/2)	50% (1/2)	[00% (1/)	33% (1/3)
T187	43% (3/7)	0% (0/4)	0% (0/2)	0% (0/2)	0% (0/)	0% (0/3)
T2S9	0% (0/7)	0% (0/4)	50% (1/2)	0% (0/2)	100% (1/)	33% (1/3)
T301	86% (6/7)	50% (2/4)	100% (2/2)	100% (2/)	100% (1/)	67% (2/3)
T311	14% (1/7)	0% (0/4)	0% (0/2)	0% (0/)	0% (0/)	0% (0/3)
Ell	43% (3/7)	50% (2/4)	0% (0/2)	100% (2/2)***	0% (0/1)	33% (1/3)
E21	14% (1/7)	50% (2/4)	50% (1/2)	0% (0/2)	100% (1/1)	33% (1/3)
El 53	29% (2/7)	25% (1/4)	0% (0/2)	0% (0/2)	0% (0/1)	33% (1/3)
Tax CTT, positives	34% (17/50)	15% (6/40)	24% (6/25)	27% (4/15)	40% (4/10)	27% (4/15)
Env CTL positives	30% (9/40)*	29% (7/24)*	7% (1/15)	22% (2/9)***	17% (1/6)	33% (3/9)**
Total CTL positives	33% (26/80)*	20% (13/64)	18% (7/40)	25% (6/24)	31% (5/16)	29% (7/24)

NT, not tested.
[a]Epitopes detected by HTLV-1/HLA tetramers/number of tetramers tested.
*$P < 0.001$.
**$P < 0.01$.
***$P < 0.05$, versus ACs.

Env21–29, and Env153–161 epitope tetramers. The epitope repertoire of HTLV-1 Env-specific $CD8^+$ cells in asymptomatic carriers showed considerably less breadth than that of HAM/TSP patients and carriers with autoimmune diseases.

Differences in Frequency of HTLV-1-Specific Tetramer Binding $CD8^+$ T Cells Among Asymptomatic HTLV-1 Carriers, HAM/TSP Patients, and Carriers With Autoimmune Diseases

There were significant differences related to clinical status with respect to the percentages of Tax-specific $CD8^+$ T cells among individuals. Among HLA-A*0201 subjects, the percentage of $CD8^+$ T cells binding Tax11–19/HLA-A*0201 tetramer in $CD8^+/CD45^+$ T lymphocytes ranged from 0.03% to 3.77% in asymptomatic HTLV-1 carriers, 0–17.1% in HAM/TSP patients, and 0–1.21% in carriers with autoimmune diseases. A similar trend was observed among HLA-A*2402 subjects, for whom the percentages of $CD8^+$ T cells binding Tax301–309/HLA-A*2402 tetramer in $CD8^+/CD45^+$ T lymphocytes ranged from 0.09% to 15.6% in asymptomatic HTLV-1 carriers, 0–26.0% in HAM/TSP patients, and 0–3.83% in carriers with autoimmune diseases. For both immunodominant HTLV-1 Tax epitopes, the mean percentage of tetramer-specific T cells within the $CD8^+$ T cell subset in HAM/TSP patients was significantly greater than in asymptomatic HTLV-1 carriers

TABLE III. The Number of Subjects Positive for HTLV-1-Specific CD8+ T Cells in Asymptomatic HTLV-1 Carriers, HAM/TSP Patients, and Carriers With Autoimmune Diseases

HLA allele	Tetramers	Asymptomatic HTLV-1 carriers	HAM/TSP	Carriers with autoimmune diseases
A*02	Tax	82% (9/11)	90% (9/10)	64% (7/11)
A*02	Env	0% (0/11)	10% (1/10)	27% (3/11)
A*24	Tax	94% (17/18)	100% (15/15)	88% (15/17)
A*24	Env	6% (1/18)	40% (6/15)*	82% (14/17)*
Tax CTL positivities		96% (25/26)	100% (18/18)	80% (20/25)
Env CTL positivities		4% (1/26)	50% (9/10)*	64% (16/25)*

*$P < 0.001$, versus ACs.

($P < 0.05\%$; Fig. 2), and carriers with autoimmune diseases ($P < 0.01$; Fig. 2), who in turn consistently had the lowest percentages of tetramer-specific $CD8^+$ T cells. HTLV-1 proviral load in HTLV-1-infected persons with autoimmune diseases was significantly lower than in asymptomatic HTLV-I carriers (28.8 and 62.2 copies/1,000 PBMCs, respectively; $P < 0.05$). With respect to Env11-19/HLA-A*2402 tetramer-binding, asymptomatic carriers consistently had the lowest percentages of Env-tetramer specific $CD8^+$ T cells (mean ± SD, 0.036% ± 0.026), which was significantly lower than in carriers with autoimmune diseases (0.067% ± 0.043, $P < 0.05$), and marginally lower than in HAM/TSP patients (0.066% ± 0.042, $P = 0.064$).

DISCUSSION

Myriad autoimmune diseases, including T cell alveolitis, myopathy, uveitis, certain types of arthritis, Sjögren's syndrome, and SLE, are seen in persons infected with HTLV-1 [Sugimoto et al., 1987; Vernant et al., 1988; Nishioka et al., 1989; Terada et al., 1994; Akimoto et al., 2007b]. The precise nature of the association between HTLV-1 and these diseases, however, remains unclear. Previous studies have focused on the rather robust cytotoxic T cell response to the HTLV-1 Tax protein in patients with HAM/TSP [Jacobson et al., 1990; Kubota et al., 2003; Bangham, 2008], but there has been little attention given to other HTLV-1

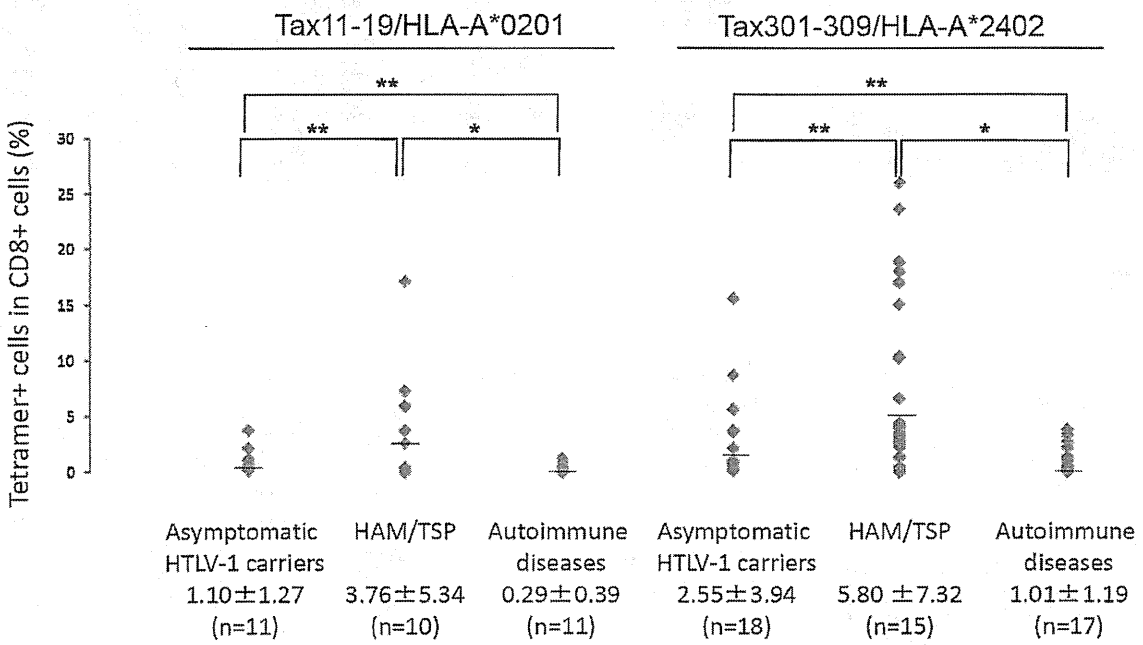

Fig. 2. Frequency of Tax11-19/HLA-A*0201 or Tax301-309/HLA-A*0201 tetramer binding $CD8^+$ T cells in asymptomatic HTLV-1 carriers, HAM/TSP patients, and carriers with autoimmune diseases. The percentage of $tetramer^+$ cells in $CD8^+$ lymphocytes in asymptomatic HTLV-1 carriers, HAM/TSP patients, and carriers with autoimmune diseases. Horizontal bars indicate the mean percentages of $tetramer^+$. The numbers below each subject group are the means ± SD. *$P < 0.01$; **$P < 0.05$ (significantly different as determined by Mann–Whitney U-test).

preferential CTL targets, particularly the HTLV-1 envelope [Pique et al., 1994, 1996]. In this study, HLA class I tetramers were used to measure the CTL response to HTLV-1 Tax and Env in a group of asymptomatic people infected with HTLV-1, patients with HAM/TSP, and persons with autoimmune diseases and concomitant HTLV-1 infection. The proportion of individuals with detectable Env tetramer-specific CTL responses was significantly higher in patients with HAM/TSP or autoimmune diseases than in asymptomatic carriers (Table II). Furthermore, patients with HAM/TSP or autoimmune diseases recognized a significantly broader repertoire of Env epitopes (four and six out of a total of six Env epitope tetramers tested, respectively), compared with asymptomatic carriers (one out of six Env epitope tetramers tested). Consistently, patients with HAM/TSP or autoimmune disease had a higher percentage of Env11-19/HLA-A*2402 specific CTLs. These findings were more pronounced for people with HLA-A*2402 than HLA-A*0201 alleles.

Antigen-mediated activation of $CD4^+$ helper T cells (Th) is essential for $CD8^+$ CTL activation. Antigen specific Th cells can be activated by Env-specific B cells, and $CD4^+$ T cells specific for intracellular viral antigens, giving cognate assistance to B cells [Scherle and Gerhard, 1986]. HTLV-1 Env epitopes eliciting both Th and B cell responses have been previously described [Jacobson et al., 1991]. Kitze et al. [1998] have also demonstrated a $CD4^+$ Th response to HTLV-1 Env glycoprotein gp21, an important target antigen in patients with HAM/TSP. The enhanced $CD4^+$ T cell responsiveness known to be characteristic of autoimmune conditions, and similarly described in HAM/TSP [Yamano et al., 1997], may therefore explain the broader Env epitope repertoire and increased Env specific CTL frequency in people with HAM/TSP or autoimmune diseases observed in this study. Previous studies examining the breadth of the T cell receptor repertoire in $CD8^+$ T cells in people infected with HTLV-1 have reported conflicting results. One study found no significant difference in the number of expanded $CD8^+$ T cell clones when asymptomatic carriers were compared with HAM/TSP patients [Eiraku et al., 1998]. Another study, using Immunoscope methods to examine the breadth of T cell clonal expansion, reported significantly greater breadth of the CTL repertoire in HAM/TSP patients compared with asymptomatic carriers [Ureta-Vidal et al., 2001]. Peripheral blood derived T lymphocytes, examined using reverse transcription-polymerase chain reaction/single-stranded conformational polymorphism methods, were also found to have a wider variety of HTLV-1 specific T-cell clonotypes in HAM/TSP patients compared with carriers [Hoger et al., 1997]. This study also demonstrates clearly, that a greater proportion of HAM/TSP patients had detectable Env specific CTLs, and that HAM/TSP patients had a broader repertoire of CTLs recognizing Env epitopes, as well as higher mean percentage of Env-specific CTLs within the $CD8^+CD45^+$ T cell subset, compared with asymptomatic carriers. This study goes further to demonstrate that like HAM/TSP patients, people with autoimmune diseases and HTLV-I infection, also have a broader CTL repertoire and higher frequency of Env-specific CTLs compared with asymptomatic carriers.

Increased proviral load in asymptomatic carriers is associated with an increased risk for progression to HAM/TSP. The Tax-specific CTL response is critical in controlling proviral load. Although increased in HAM/TSP compared with asymptomatic carriers [Nagai et al., 1998], the CTLs in HAM/TSP have been demonstrated to have lower cytolytic efficiency [Bangham, 2008; Kattan et al., 2009]. The higher Tax-specific CTL frequency, but with higher proviral load, observed in HAM/TSP patients compared with asymptomatic persons infected with HTLV-1 is consistent with low CTL efficiency. Conversely, higher CTL efficiency could explain the lower $CD8^+$ Tax-specific CTL frequency seen in people with autoimmune diseases, who have a significantly lower proviral load than asymptomatic carriers in this study. This difference in CTL efficiency remains to be confirmed.

In this study, it was demonstrated that the difference in frequency of HTLV-1 Tax-specific $CD8^+$ T cells was not statistically significant between asymptomatic HTLV-1 carriers, HAM/TSP or carriers with autoimmune diseases, while HTLV-1 Env-specific $CD8^+$ T cells were significantly more frequent in HAM/TSP and carriers with autoimmune diseases than those in asymptomatic HTLV-1 carriers. These results suggest that the diversity, frequency, and repertoire of HTLV-1-specific $CD8^+$ T cell clones, especially HTLV-1 Env $CD8^+$ T cells may be related to the hyperimmune response in HAM/TSP and carriers with autoimmune diseases, although different immunological mechanisms may mediate the hyperimmune responses in HAM/TSP and autoimmune diseases.

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Programmed death-1 (PD-1)/PD-1 ligand pathway–mediated immune responses against human T-lymphotropic virus type 1 (HTLV-1) in HTLV-1-associated myelopathy/tropical spastic paraparesis and carriers with autoimmune disorders

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ABSTRACT

Human T-lymphotropic virus-1 (HTLV-1) causes HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and adult T-cell leukemia–lymphoma in individuals with dysfunctional immune responses. In this study, to characterize the HTLV-1-specific cytotoxic T lymphocyte (CTL) populations in asymptomatic HTLV-1 carriers (ACs), HAM/TSP patients, and carriers with autoimmune disorders (CAIDs), we examined the role of programmed death-1 and its ligand (PD-1/PD-L1) in HTLV-1-specific CTL functions using an HTLV-1 Tax/HLA-A*0201 tetramer and an HTLV-1 Tax/HLA-A*2402 tetramer. Interestingly, the percentage of HTLV-1 Tax301–309/HLA-A*2402 tetramer$^+$CD8$^+$ cells expressing PD-1 in ACs was significantly higher than the percentage of HTLV-1 Tax11–19/HLA-A*0201 tetramer$^+$CD8$^+$ cells expressing PD-1. PD-1 expression was significantly downregulated on HTLV-1-specific CTLs in HAM/TSP compared with ACs. PD-L1 expression was observed in a small proportion of unstimulated lymphocytes from ACs and was greater in ACs than in HAM/TSP and CAIDs after short-term culture. Furthermore, CTL degranulation was impaired in HAM/TSP, whereas anti-PD-L1 blockade significantly increased CTL function in ACs. Downregulation of PD-1 on HTLV-1-specific CTLs and loss of PD-L1 expression in HAM/TSP and CAIDs, along with impaired function of HTLV-1-specific CTLs in HAM/TSP, may underlie the apparently dysfunctional immune response against HTLV-1.

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1. Introduction

Human T-lymphotropic virus-1 (HTLV-1) causes HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and adult T-cell leukemia–lymphoma (ATL) after long-term infection [1–4]. The immune response to HTLV-1 is typically enhanced in HAM/TSP, whereas impaired cell-mediated immunity has been implicated in ATL [5–7]. A variety of autoimmune-like disorders are seen in HTLV-1-infected individuals [8], although the precise relationship between these disorders and HTLV-1 infection remains unclear [9–11].

Natural infection of goats with caprine arthritis encephalitis virus results in an arthropathy that mimics human rheumatoid arthritis (RA) [12]. HTLV-1 infection is a risk factor for the development of RA [13]. Endogenous murine retroviruses are involved in the etiology of a systemic lupus erythematosus (SLE)-like disease in specific inbred strains of mice [14]. Indeed, patients with SLE and concomitant HTLV-1 infection seem to have a more indolent clinical course compared with that of patients with SLE who are not infected with HTLV-1 [15]. Transgenic mice carrying retrovirus-specific genes, including HTLV-1 Tax, show autoimmune-like pathology, suggesting that these viruses have the potential to induce autoimmune disorders [16,17]. However, HTLV-1 is also associated with polyarthritis and proliferative synovitis, known as HTLV-1-associated chronic inflammatory arthropathy, in some HTLV-1 seropositive individuals [18,19]. In addition, there is serologic evidence linking HTLV-1 to Sjögren's syndrome (SS). In the Nagasaki region of Japan, where HTLV-1 is endemic, there is a high prevalence of anti-HTLV-1 antibodies, and 36% of SS patients are positive for these antibodies [20]. In another study from the same region, SS patients exhibited high serum reactivity to HTLV-1 (23%) compared with only 3% of the general population [10] and a high prevalence of HAM/TSP [21,22]. HTLV-1 infection may also play a role in the etiology of animal autoimmune disorders, and the pathogenesis of these diverse autoimmune conditions may be similar, but still different from HAM/TSP. At present, it is not known whether the HTLV-1 Tax-specific cytotoxic T lymphocytes (CTLs) involved in

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HAM/TSP are similar to, or different from, those observed in HTLV-1-associated autoimmune disorders.

Negative immunoregulatory programmed death-1 (PD-1) signaling is involved in autoimmunity, allergy, sites of immune privilege, and antitumor immunity [23–26]. Sharpe et al. demonstrated that PD-1/PD-1 ligand (PD-L1) interactions control the induction and maintenance of peripheral T-cell tolerance and indicated a previously unknown function of PD-L1 on nonhematopoietic cells in protecting tissues from autoimmune attack. The PD-1/PD-L1 pathway may also be exploited by a variety of microorganisms to attenuate antimicrobial immunity and facilitate chronic infection [25].

We previously reported on the repertoire, function, and upregulation of PD-1 expression on HTLV-1-specific CTLs in asymptomatic HTLV-1 carriers (ACs) and ATL patients [27,28]. This increased PD-1 expression suggests that PD-1 signaling plays a role in fostering persistent HTLV-1 infection, facilitating immune evasion by ATL cells, and thereby furthering ATL development [29]. However, despite recognition of the PD-1/PD-L1 pathway in immune dysregulation, there are no reports regarding the PD-1 expression of HTLV-1-specific CTL in diseases related to HTLV-1-host immune responses, such as HAM/TSP or other autoimmune disorders.

Both HAM/TSP and autoimmune disorders may be associated with host immune responses to the virus in HTLV-1-infected individuals [8]. The aim of this study was to characterize HTLV-1-specific CTLs in HTLV-1-infected subjects with dysfunctional immune responses, such as HAM/TSP patients and carriers with autoimmune disorders (CAIDs), and compare them with those from ACs. Therefore, we examined PD-1 expression by HLA-A*0201- or HLA-A*2402-restricted HTLV-1 Tax tetramer-specific CTLs in these groups and the effects of PD-L1 blockade on CTL function.

2. Subjects and methods

2.1. Subjects

The subjects enrolled in this study comprised 20 ACs (24–81 years old, mean = 58.3), 30 HAM/TSP patients (34–73 years old, mean = 54.6), and 15 HTLV-1-infected patients with autoimmune disorders (32–80 years old, mean = 59.3), all of whom were recruited through the Kagoshima University Hospital. Patients seropositive for HTLV-1, but without clinical symptoms of HTLV-1-related diseases, were designated ACs. Autoimmune disorders included 7 patients with SS, 5 with SLE, and 3 with RA. The study protocol was reviewed and approved by the Medical Ethical Committee of Kagoshima University.

2.2. Phenotypic analysis

Phenotypic analysis of peripheral blood mononuclear cells (PBMCs) using an HTLV-1 Tax/HLA-A*0201 tetramer, an HTLV-1 Tax/HLA-A*2402 tetramer, a CMV pp65 (QYDPVAALF)/HLA-A*2402 tetramer, an EBV BRLF1 (TYPVLEEMF)/HLA-A*2402 tetramer (Medical and Biological Laboratories, Nagoya, Japan), anti-PD-1 mAbs (eBioscience, San Diego, CA), and anti-PD-L1 monoclonal antibodies (mAbs; MIH1; eBioscience) was performed as previously described [30–32]. PBMCs were screened by serologic staining with mAbs against HLA-A*02 supertype (clone BB7.2) and HLA-A*24 supertype (clone 17A10) (Medical and Biological Laboratories), followed by secondary staining with goat antimouse IgG-FITC (Immunotech, Miami, FL). The cells were then analyzed by flow cytometry using a FACScan cytometer (BD Biosciences, Mountain View, CA). Subjects expressing neither HLA-A*02 nor HLA-A*24 were excluded from the study.

2.3. Intracellular Tax staining assay

PBMCs used for HTLV-1 Tax and PD-L1 expression analysis were cultured for 12 hours and stained as previously described [28]. Briefly, PBMCs (1×10^6) were cultured for 12 hours in complete medium (CM; RPMI 1640 supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.05 mM 2-mercaptoethanol, 50 U/mL recombinant human interleukin-2, and 10% heat-inactivated fetal calf serum). For cell-surface antigen analysis, PBMCs were labeled with Cy7-conjugated murine anti-PD-L1 mAbs, anti-CD4-PE, and anti-CD25-allophycocyanin antibody (Becton Dickinson, Mountain View, CA). These cells were further treated with permeabilizing solution (Becton Dickinson). After being washed, the cells were incubated with anti-Tax-FITC (clone Lt4; kindly provided by Y. Tanaka, Ryukyu University, Okinawa, Japan). As a negative control, staining was also performed with an isotype control IgG1-FITC for Tax (Becton Dickinson). Lymphocyte analysis was performed using a FACSCalibur (Becton Dickinson) and data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

2.4. CD107a mobilization assay

Assessment of cytolytic ability in the presence of a blocking antibody specific to PD-L1 was performed using flow cytometric quantification of the surface mobilization of CD107a as a marker of degranulation after stimulation, as previously described [27,28]. Briefly, PBMCs (1×10^6) were cultured for 6 hours in CM with or without 0.02 μM HTLV-1 Tax peptide (Sigma–Aldrich, Tokyo, Japan) in combination with anti-CD107a mAb-FITC (clone H4A3; Southern Biotech, Birmingham, AL) and the secretion inhibitor monensin (Becton Dickinson). A blocking antibody specific to PD-L1 (clone MIH1; eBioscience) was added to the cell cultures at a concentration of 10 μg/mL. The cells were further stained with HLA-tetramer-PE and anti-CD8 mAb-PE/Cy5 (Beckman Coulter) as previously described [28]. Aliquots of 1×10^4 $CD8^+$ T lymphocytes were examined using a FACSCalibur and data were analyzed using FlowJo software.

2.5. Statistical analysis

The Mann–Whitney *U* test and the Wilcoxon matched pairs test were performed using StatView software version 5.0 (SAS Institute, Inc., Cary, NC). p values < 0.05 were considered significant.

3. Results and discussion

3.1. Frequency and PD-1 expression on the HTLV-1-specific tetramer$^+$ cells within the CD8$^+$ lymphocyte population

The frequency of $CD8^+$ T cells binding the Tax301–309/HLA-A*2402 tetramer ranged from 0.09 to 8.64% (2.78 ± 3.74) in AC and from 0 to 26.6% (7.56 ± 8.08) in HAM/TSP. Binding was significantly less in CAIDs compared with that in ACs and HAM/TSP, ranging from 0 to 2.73% (0.76 ± 0.82; $p < 0.05$ and $p < 0.01$, respectively; % ± SD; Table 1). A similar trend was observed for the HLA-A*0201 tetramer in ACs, HAM/TSP, and CAIDs [32], whereas the frequency of HTLV-1 Tax301–309/HLA-A*2402 tetramer$^+$$CD8^+$ cells tended to be lower than that of HTLV-1 Tax11–19/HLA-A*0201 tetramer$^+$$CD8^+$ cells. The frequency of $CD8^+$ T cells binding the Tax11–19/HLA-A*0201 tetramer was 1.65 ± 1.43% in AC, 4.99 ± 5.38% in HAM/TSP, and 0.40 ± 0.47% in CAIDs (data not shown). Furthermore, the percentage of HTLV-1 Tax301–309/HLA-A*2402 tetramer$^+$$CD8^+$ cells expressing PD-1 in ACs was significantly higher than the frequency of HTLV-1 Tax11–19/HLA-A*0201

Table 1
The percentage of tetramer$^+$ cells within the $CD8^+$ lymphocyte populations from ACs, HAM/TSP patients, and CAIDs

	AC	HAM/TSP	CAIDs
HTLV-1 Tax/HLA-A*2402 Tetramer$^+$ cells in $CD8^+$ cells	2.78 ± 3.74% ($N = 20$)	7.56 ± 8.08%* ($N = 29$)	0.64 ± 0.81%* ($N = 15$)

Results represent the mean ± SD.
*$P < 0.05$ versus AC.