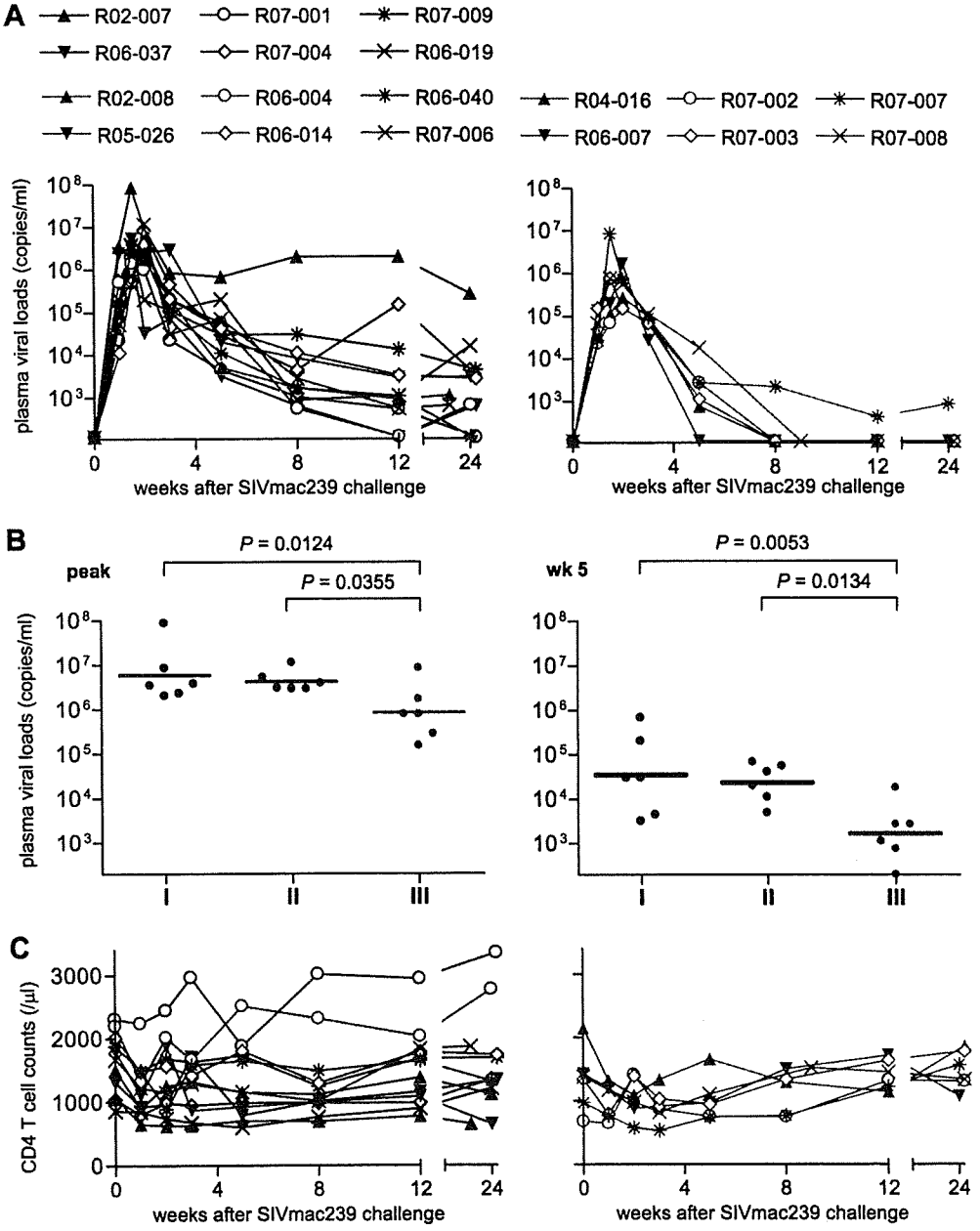

FIG. 2. Plasma viral loads and peripheral CD4$^+$ T-cell counts after a SIV challenge. (A) Changes in plasma viral loads (SIV *gag* RNA copies/ml plasma) in unvaccinated group I animals (black lines in the left panel), control-vaccinated group II animals (blue lines in the left panel), and $Gag_{236\text{-}250}$-vaccinated group III animals (red lines in the right panel) after a SIVmac239 challenge. Plasma viral loads were determined as described previously (27). The lower limit of detection is approximately 4×10^2 copies/ml. (B) Comparisons of plasma viral loads in groups I ($n = 6$), II ($n = 6$), and III ($n = 6$) at the peak (left panel) and at week 5 (right panel). The bar indicates the geometric mean of each group. Viral loads at the peak and at week 5 in group III were significantly lower than in group I ($P = 0.0124$ at the peak and $P = 0.0053$ at week 5) and group II ($P = 0.0355$ at the peak and $P = 0.0134$ at week 5). There were no significant differences between groups I and II either at the peak or at week 5 ($P = 0.6047$ at the peak and $P = 0.6536$ at week 5). Set point viral loads in group III were significantly lower than those in group I and group II at week 12 by nonparametric analysis ($P = 0.3939$ between I and II, $P = 0.0152$ between I and III, and $P = 0.0152$ between II and III; $P = 0.1797$ between I and II, $P = 0.0260$ between I and III, and $P = 0.0411$ between II and III around week 24). (C) Changes in peripheral CD4$^+$ T-cell counts (per μl) in groups I (black lines) and II (blue lines) in the left panel and in group III (red lines) in the right panel after a SIVmac239 challenge.

$Gag_{241\text{-}249}$-specific induction of CD107a (a degranulation marker), which is related to cytolytic activity (21, 38), in CD8$^+$ T cells at week 2. Frequencies of CD8$^+$ T cells exhibiting $Gag_{241\text{-}249}$-specific induction of CD107a, as well as IFN-γ, within the CD8$^+$ T-cell pool were significantly higher in group III than in naive controls ($P = 0.0249$ by unpaired t test) (Fig. 6). One animal, R04-016, in group III did not show $Gag_{241\text{-}249}$-specific CD107a$^+$ IFN-γ$^+$ CD8$^+$ T-cell responses, but further

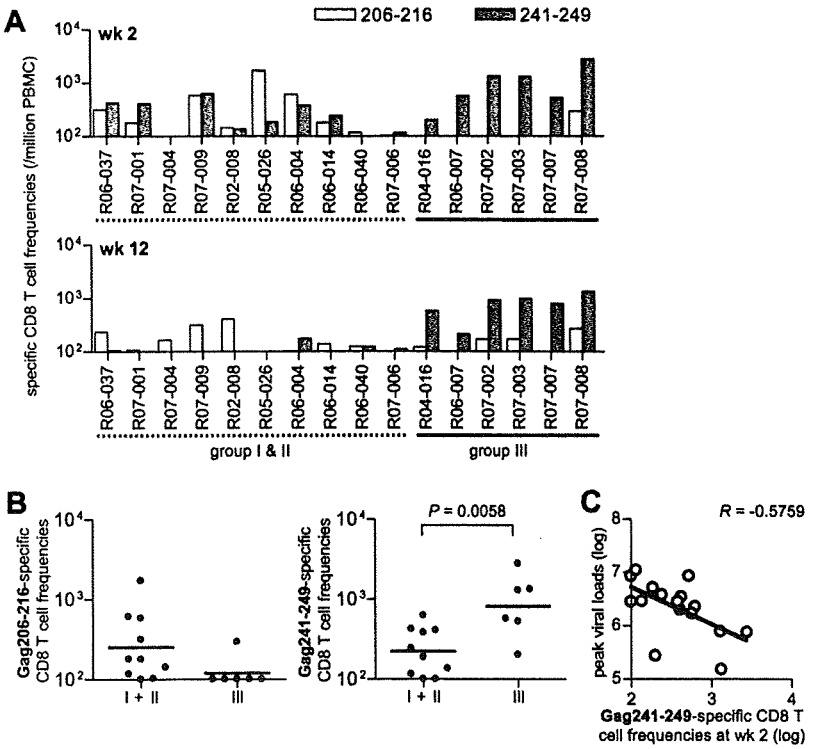

FIG. 3. Gag epitope-specific $CD8^+$ T-cell frequencies after a SIV challenge. (A) Frequencies of $CD8^+$ T cells (per million PBMCs) showing $Gag_{206-216}$-specific (open boxes) or $Gag_{241-249}$-specific (closed boxes) IFN-γ induction in naive controls and group III macaques at week 2 (upper panel) and week 12 (lower panel). (B) Comparison of the $Gag_{206-216}$-specific (left panel) or $Gag_{241-249}$-specific (right panel) $CD8^+$ T-cell frequencies in naive controls ($n = 10$) and group III animals ($n = 6$) at week 2. The bar indicates the geometric mean of each group. Frequencies of $Gag_{241-249}$-specific ($P = 0.0058$) but not $Gag_{206-216}$-specific ($P = 0.0922$) $CD8^+$ T cells in group III were significantly higher than in naive controls. The $Gag_{241-249}$-specific frequencies at week 12 in group III were significantly higher than those in naive controls ($P < 0.0001$). (C) Analysis of the correlation between $Gag_{241-249}$-specific $CD8^+$ T-cell frequencies (log) at week 2 and peak plasma viral loads (log). An inverse correlation is shown ($P = 0.0196$, $R = -0.5759$). Samples from macaques R02-007 and R06-019 in group I were unavailable for this analysis.

analysis revealed that this animal had $Gag_{241-249}$-specific granzyme B^+ IFN-γ^+ $CD8^+$ T cells. Indeed, group III animals had significantly higher frequencies of $Gag_{241-249}$-specific IFN-γ^+ $CD8^+$ T cells producing CD107a, granzyme B, or perforin ($P = 0.0076$; data not shown). These results indicate efficient induction of $Gag_{241-249}$-specific $CD8^+$ T cells with higher cytolytic activity in the acute phase in group III animals.

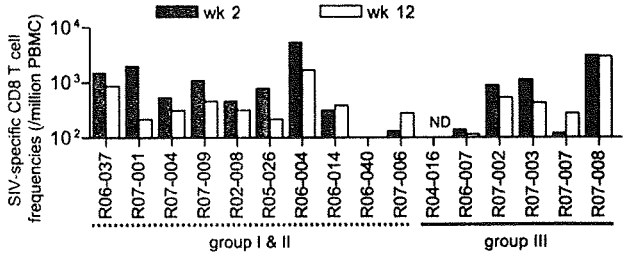

FIG. 4. SIV-specific $CD8^+$ T-cell frequencies after a SIV challenge. SIV-specific $CD8^+$ T-cell frequencies (per million PBMCs) in naive controls and group III macaques at week 2 (closed boxes) and week 12 (open boxes) are shown. ND, not determined.

DISCUSSION

In the present study, induction of $CD8^+$ T cells specific for a single $Gag_{241-249}$ epitope by prophylactic vaccination resulted in a significant reduction of plasma viral loads after a SIV challenge. Even if vaccines are designed to express multiple antigens, of the vaccine-induced $CD8^+$ T cells generated, at most one or only a few epitope-specific cells may recognize the incoming HIV because of viral diversity and host MHC polymorphisms (10). Our finding, however, implies that even a $CD8^+$ T-cell memory response to a single epitope which can recognize the incoming HIV could facilitate HIV control.

Group III macaques showed more effective $CD8^+$ T-cell responses than did naive controls after a SIV challenge. Our previous trial of a vaccine inducing Gag-specific T-cell responses resulted in SIV control in *90-120-Ia*-positive macaques with rapid selection of the GagL216S mutation escaping from $Gag_{206-216}$-specific $CD8^+$ T-cell recognition at week 5 (27). In contrast, the $Gag_{236-250}$ vaccination resulted in SIV control without *gag* mutation selection over 5 weeks in the present study, reflecting the fact that, rather than $Gag_{206-216}$-specific $CD8^+$ T-cell responses, dominantly induced $Gag_{241-249}$-specific $CD8^+$ T-cell responses played a central role in the reduc-

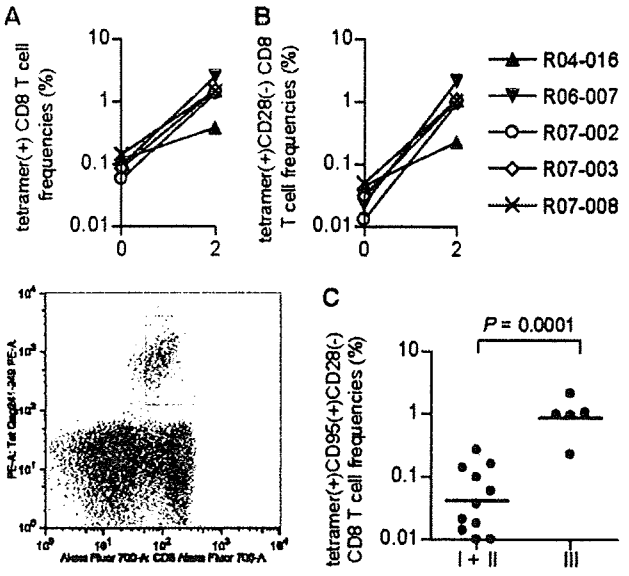

FIG. 5. Frequencies of $Gag_{241-249}$-specific $CD8^+$ T cells detected by $Gag_{241-249}$-Mamu-A*90120-5 tetramers after a SIV challenge. (A) Frequencies of $Gag_{241-249}$-Mamu-A*90120-5 tetramer$^+$ cells within $CD8^+$ T cells in group III animals before a challenge (week 0) or at week 2 after a challenge. A representative dot plot gated on $CD3^+$ lymphocytes for determining tetramer$^+$ $CD8^+$ T cells (x axis, CD8; y axis, tetramer) in macaque R07-008 is shown in the lower panel. (B) Tetramer$^+$ $CD28^-$ cell frequencies in $CD8^+$ T cells in group III animals at weeks 0 and 2. Data on tetramer$^+$ $CD95^+$ $CD28^-$ $CD8^+$ T-cell frequencies at week 0 are unavailable. (C) Tetramer$^+$ $CD95^+$ $CD28^-$ $CD8^+$ T-cell frequencies in naive controls (groups I and II) and group III animals at week 2. The bar indicates the geometric mean of each group. The frequencies in group III were significantly higher than those in naive controls (P = 0.0001 by unpaired t test). Samples from macaques R06-019 in group I and R07-007 in group III were unavailable for this analysis.

tion of viral loads in the acute phase. These results suggest that this vaccination approach altered the dominance pattern of $CD8^+$ T-cell responses and resulted in dominant induction of effective $Gag_{241-249}$-specific $CD8^+$ T-cell responses in the acute phase after a SIV challenge, facilitating a reduction in peak viral loads. Selection of vaccine epitopes for induction of $CD8^+$ T-cell responses might be important for viral control because the antiviral efficacy of $CD8^+$ T cells could be affected by MHC-I-restricted target epitopes (10, 19, 25, 35).

$Gag_{241-249}$-specific $CD8^+$ T-cell induction by prophylactic vaccination resulted in higher frequencies of these T-cell responses during the acute phase after the SIV challenge. The induction of $Gag_{241-249}$-specific effector memory $CD8^+$ T cells was especially marked. We did not examine polyfunctionality, but analyses of a cytolytic marker, CD107a, indicated higher frequencies of $Gag_{241-249}$-specific cytolytic $CD8^+$ T-cell responses, implying that these T cells originating from vaccine-induced memory may have higher cytolytic activity in the acute phase. These results suggest that group III animals with $Gag_{241-249}$-specific memory $CD8^+$ T cells showed induction of a high magnitude of $Gag_{241-249}$-specific $CD8^+$ T cells with effector function after a SIV challenge, resulting in reduction of viral loads in the acute phase.

In this study, some *90-120-Ia*-positive unvaccinated macaques showed lower viral loads. However, in our previous studies with Burmese rhesus macaques (reference 15 and unpublished data), all unvaccinated *90-120-Ia*-negative animals failed to contain a SIVmac239 challenge and animals, including vaccinees, that failed to control SIVmac239 replication developed AIDS in 1 to 4 years; even R-90-120 descendants possessing the MHC-I haplotype *90-120-Ib* but not *90-120-Ia* (both *90-120-Ia* and *90-120-Ib* are derived from breeder R-90-120) showed high viral loads. Additionally, *90-120-Ia*-positive animals failed to control the replication of SIVmac239 carrying CTL escape mutations (16). Thus, a SIVmac239 challenge of Burmese rhesus macaques mostly results in persistent viremia and progression to AIDS but some *90-120-Ia*-positive animals may show lower viral loads due to *90-120-Ia*-associated SIV-specific CTL responses. However, a previously reported *90-120-Ia*-positive unvaccinated macaque, R02-007, developed AIDS around 3 years after a SIVmac239 challenge. Furthermore, two of the *90-120-Ia*-positive vaccinees that controlled a SIVmac239 challenge but showed reappearance of viremia

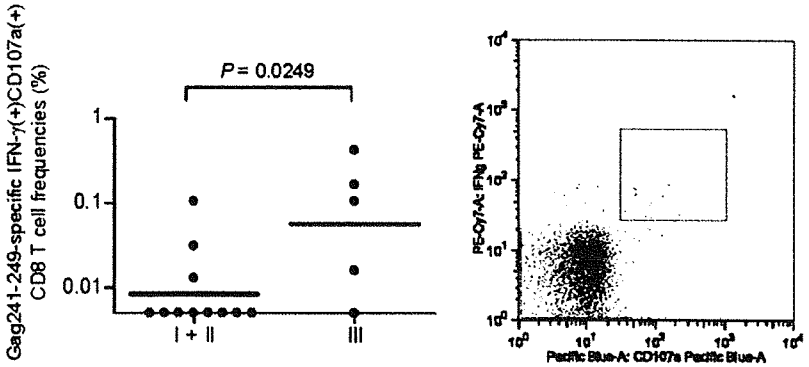

FIG. 6. $Gag_{241-249}$-specific cytolytic $CD8^+$ T-cell frequencies at week 2 after a challenge. PBMCs were cultured in the absence or the presence of the $Gag_{241-249}$ peptide for unstimulated controls or $Gag_{241-249}$-specific stimulation, and the frequencies of $CD8^+$ T cells exhibiting $Gag_{241-249}$-specific induction of both IFN-γ and CD107a in the total $CD8^+$ T cells were examined. The bar indicates the geometric mean of each group. The frequencies in group III were significantly higher than those in naive controls (P = 0.0249 by unpaired t test). The right panel is a representative dot plot showing the CD107a (x axis) and IFN-γ (y axis) responses in $CD8^+$ T cells in macaque R07-008 after $Gag_{241-249}$-specific stimulation. Samples from macaques R06-019 in group I and R07-007 in group III were unavailable for this analysis.

around 1 year later developed AIDS (15). Thus, it is inferred that the majority of *90-120-Ia*-positive unvaccinated macaques develop AIDS after a SIVmac239 challenge. Several MHC-I alleles are known to be associated with lower viral loads in HIV and SIV infections, and potent CTLs directed against these MHC-I-restricted epitopes have been implicated in the suppression of viral replication (7, 8, 9, 10, 13, 18, 22, 31, 33, 48). The $Gag_{241-249}$-specific CTL may also be naturally potent (10, 16), but the impact of memory induction of even these potent CTLs on viral control has not yet been determined. Thus, this is the first study documenting the benefit of single-epitope-specific memory $CD8^+$ T-cell induction by prophylactic vaccination for HIV/SIV control. Further analysis with a vaccine expressing a single helper epitope, as well as a CTL epitope, would contribute to evaluation of the impact of HIV/SIV-specific $CD4^+$ T-cell memory induction on HIV/SIV replication.

Because $CCR5^+$ memory $CD4^+$ T cells, especially HIV-specific $CD4^+$ T cells, are themselves targets of this virus, whether virus-specific $CD4^+$ T-cell induction by prophylactic vaccination can result in effective virus-specific $CD4^+$ T-cell responses postinfection and contribute to HIV control remains unclear. On the other hand, it has been unknown whether HIV-specific memory $CD8^+$ T cells induced by vaccination without HIV-specific $CD4^+$ T-cell help can elicit effective responses after virus exposure. In the present study, the $pGag_{236-250}$-EGFP/F(−)SeV-$Gag_{236-250}$-EGFP vaccination elicited $Gag_{241-249}$-specific $CD8^+$ T-cell responses without SIV-specific $CD4^+$ T-cell help but possibly with EGFP-specific or SeV-specific $CD4^+$ T-cell help; i.e., SeV-EGFP-specific $CD4^+$ T cells would confer cognate help for $Gag_{241-249}$-specific $CD8^+$ T-cell induction. The $Gag_{241-249}$-specific memory $CD8^+$ T cells induced by prophylactic vaccination without SIV-specific $CD4^+$ T-cell help but with non-SIV-specific $CD4^+$ T-cell responses responded efficiently to a SIV challenge, showing dominant $Gag_{241-249}$-specific $CD8^+$ T-cell responses resulting in SIV control; infection-induced SIV-specific $CD4^+$ T-cell responses may be involved in $Gag_{241-249}$-specific $CD8^+$ T-cell induction postinfection. Therefore, this study documents that prophylactic vaccination eliciting virus-specific $CD8^+$ T-cell memory even without virus-specific $CD4^+$ T-cell responses (but with cognate non-virus-specific $CD4^+$ T-cell responses) can facilitate SIV control after a challenge.

Taken together, the present study demonstrates that induction of single-epitope-specific $CD8^+$ T-cell memory without virus-specific $CD4^+$ T-cell help by prophylactic vaccination can result in dominant potent $CD8^+$ T-cell responses and control of SIV replication after a challenge. These results imply possible HIV control by prophylactic vaccination eliciting virus-specific $CD8^+$ T-cell memory with non-virus-specific $CD4^+$ T-cell help and provide valuable insights into AIDS vaccine development.

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特集2　ワクチンの現状と将来

4. エイズワクチン開発の論理

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ヒト免疫不全ウイルス（HIV）感染症では，自然感染によって誘導される宿主免疫反応によってもウイルスが十分には排除されず，慢性持続感染が成立し，その結果，エイズ発症にいたる．主に自然感染の模倣という概念でワクチン開発が成功してきた急性感染症に対し，このような自然治癒に至らない感染症に対するワクチン開発においては従来の枠を超えた新たな戦略が必要となる．これまでの多大な努力により，技術開発および臨床試験体制構築に進展がみられているものの，感染予防効果を有するエイズワクチン開発には至っていない．結局，どのような免疫反応を誘導すればHIV感染・複製阻止に結びつくかということを明らかにすることが最重要課題となっている．本稿では，適応免疫の代表的エフェクターである抗体と細胞傷害性Tリンパ球の作用に着目し，ワクチンで誘導されるエフェクターとメモリーが，HIV曝露に対しどのような影響を及ぼすかという視点からワクチン開発の論理を考察する．さらに，国際臨床試験計画が進展している我々のエイズワクチンシステムの位置づけを簡単に解説する．

1. はじめに

1981年に後天性免疫不全症候群（エイズ）の症例が報告され，その後，ヒト免疫不全ウイルス（HIV）がその病原ウイルスであることが判明してから四半世紀余りが経過しているが，世界のHIV感染者数の増加は今なお続いている．UNAIDS（Joint United Nations Programme on HIV/AIDS, http://www.unaids.org/）の推定では，2007年末の時点で，世界のHIV感染者数は約3300万人，1年間の新規感染者数は約250万人，1年間のエイズによる死亡者数は約210万人であり，わが国でも2008年には過去最多の1100人あまりの新規HIV感染者が報告されている（厚生労働省エイズ動向委員会）．アフリカを中心とした流行地域でのHIV感染拡大は，HIVに増殖・変異の場を与え，宿主免疫反応による抑制をよりうけにくいHIVの出現に結びつく可能性や，先進国で奏効している抗HIV薬に対する耐性変異株出現に結びつく可能性が危惧されている．さらに，免疫能が低下傾向にあるHIV感染者数の増加により新興再興感染症出現を促進する危険性も考えられる．したがって，HIV感染拡大は，流行地域だけではなくグローバルな視点で取り組み克服すべき国際的重要課題であり，ウイルス増殖の場を減らすという予防戦略がHIV感染症克服の基本となる．

感染拡大阻止のためには，まず社会的予防活動が基本となるが，HIV感染症のように無症候期の長い慢性感染症では，感染者の隔離も難しく，社会的予防活動だけによる封じ込めには限界がある．そのため，HIV感染拡大阻止の切り札として，予防エイズワクチン開発は鍵となる戦略である．予防エイズワクチンにより，ある程度の感染拡大抑制効果が得られれば，社会的予防活動と抗HIV薬などによるHIV感染症のコントロールが期待できる．つまり，この予防エイズワクチン開発は，主対象であるHIV感染流行地域での感染拡大阻止を介して，流行地域以外も含めた世界全体のHIV感染症克服に結びつくという認識である．

2. HIV感染症の特徴

HIV感染症の自然経過では，誘導される適応免疫反応によってもHIV複製が制御されず持続感染が成立し，最終的

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にエイズ発症に至る．したがって，エイズワクチン開発においては，従来のワクチンのような自然感染の模倣という考え方だけでは通用せず，新しい概念に基づく戦略が必要となる．まずはHIV持続感染成立を阻止する防御機序の解明が重要であり，これまで，特に適応免疫の代表的エフェクターである抗体と細胞傷害性Tリンパ球（CTL）に焦点をおいた研究が進められてきた．HIV感染症の自然経過における適応免疫反応の特徴は以下のとおりである．

(1) 特にHIV感染急性期において，HIVの細胞侵入を阻害する中和抗体の誘導効率が悪いことが知られている[1]．
(2) サルエイズモデルにおける中和抗体受動免疫実験により[2,3]，慢性持続感染成立後に誘導される中和抗体は，個体レベルでは強い体内HIV増殖抑制効果を呈さないと考えられている．ただし，中和抗体に対するエスケープ変異が選択されることから，ある程度の抑制圧を有していることが示唆されている[4,5]．
(3) ヒトHIV感染急性期における解析（体内ウイルス量とHIV特異的CTLレベルとの逆相関）[6,7]ならびにサルエイズモデルにおけるCD8陽性細胞枯渇実験により[8,9]，CTLは強い体内HIV増殖抑制効果を有し，持続感染成立阻止には至らないもののHIV複製抑制に中心的役割を果たしていることが示されている．特に，Gag抗原特異的CTLが強いHIV複製抑制効果を有することが示唆されている[10]．
(4) CTLは，主要組織適合遺伝子複合体クラスI（MHC-I）に結合してHIV感染細胞表面上に提示されるHIV抗原由来ペプチド（エピトープ）を特異的に認識する．このMHC-I（ヒトではHLA）遺伝子型が，HIV感染者の体内ウイルス量に大きく影響を与えることが知られており，急性発症に結びつく傾向が高いHLA遺伝子型や，長期未発症に結びつく傾向が高いHLA遺伝子型が報告されている[11,12]．これらの知見は，CTLの強いHIV複製抑制効果を反映している．

したがって，HIV自然感染では，CTL反応によるHIV複製抑制は認められるものの持続感染成立に至るということになる．その主な原因として，「感染急性期の中和抗体誘導が不十分であること」および「誘導されるCTLによるHIV複製抑制が不十分であること」の2点が考えられ，これらの克服を模索することは，ワクチン開発における重要な戦略である．

これらに関連するHIVの特徴として，その感染の主標的がCCR5陽性CD4陽性Tリンパ球であることが挙げられる．そのため，HIV感染急性期に，腸管を中心とするメモリーCD4陽性Tリンパ球の大量破壊に至ることが報告されており，このことがその後の感染慢性期の免疫活性化を経てエイズ発症に結びつく第一歩となっていると考えられている[13-17]．特に，急性期のCD4陽性ヘルパーTリンパ球機能不全を介して，CTLの機能不全に結びついている可能性がある．実際，CCR5陽性CD4陽性Tリンパ球を主標的とするサル免疫不全ウイルス(SIV)あるいはSIV-HIVキメラウイルス(SHIV)感染サルは慢性持続感染を呈するのに対し，CXCR4陽性CD4陽性Tリンパ球を主標的とするSHIV感染は急性感染を呈し，ワクチンによるコントロールも比較的容易である[18,19]．さらに，前者の中和抗体誘導不全の主因としては，HIVエンベロープ（Env）抗原の構造上の特殊性（高度に糖鎖修飾されていることやレセプター結合部位に抗体がアクセスしにくいことなど）[1,4,20]が挙げられ，後者のCTLについては，HIVのCTL存在下における複製能力が関与している可能性も考えられる．

3. HIV感染・複製阻止に結びつく免疫機序

上記の自然感染における知見をもとに，次のステップとして，ワクチンによる免疫誘導がHIV曝露時あるいは曝露後のHIV感染・複製にどのような防御効果を発揮しうるかということを考える必要がある．このステップにおいては，サルエイズモデルにおける解析が重要な役割を担っている．ワクチンは免疫メモリーを誘導するという概念が一般的であるが，最も標準的なワクチンのイメージは，エフェクターとしての抗体誘導によるウイルス感染阻止である．しかし，急性感染症におけるこのような抗体による感染阻止とされる現象も，抗体（エフェクター）による感染阻止だけではなく，感染後のメモリーリンパ球の2次反応によるウイルス増殖抑制も働いて発症阻止に結びついている可能性がある．

これらの点を考慮し，ワクチンにより誘導される適応免疫のエフェクター的要素とメモリー的要素を整理して考えてみると，ワクチンによるHIV感染・複製阻止に結びつく機序として，以下のようにまとめることができる（**図1**）．まず，中和抗体誘導を主眼とするワクチンでは，(A) HIV曝露時に既に存在する中和抗体（エフェクター）によるHIV感染防御の可能性と，(B) HIV曝露後のメモリーBリンパ球の2次反応に起因する抗体反応によるHIV複製抑制の可能性が考えられる．一方，CTL誘導を主眼とするワクチンでは，(C) HIV曝露時に既に存在する（エフェクターあるいは）エフェクターメモリー（EM）CTL反応によるHIV複製抑制の可能性と，(D) HIV曝露後のセントラルメモリー（CM）CTLの2次反応によるHIV複製抑制の可能性が考えられる．なお，いずれにおいてもワクチンで誘導されるメモリーCD4陽性Tリンパ球反応の関与も検討課題となる．

3A. ワクチン誘導中和抗体によるHIV感染防御の可能性

中和抗体による感染防御はワクチンの標準的な概念であり，以前より，この機序に沿った試みが数多くなされてきた．この機序によるHIV感染抑制の可能性については，サルエイズモデルにおけるSIVあるいはSHIVチャレンジ前

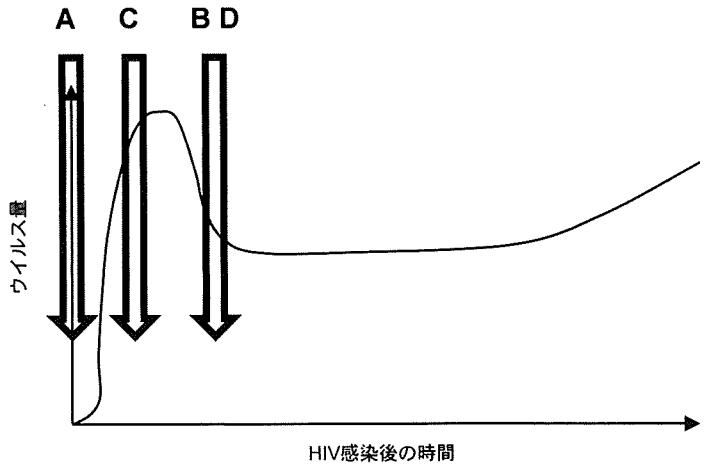

図1 ワクチンによるHIV感染・複製抑制機序
(A) HIV曝露時の中和抗体によるHIV感染防御.
(B) HIV曝露後のメモリーBリンパ球反応・中和抗体反応によるHIV複製抑制.
(C) HIV曝露時・曝露直後のエフェクターメモリーCTL反応によるHIV複製抑制.
(D) HIV曝露後のセントラルメモリーCTL反応によるHIV複製抑制.

の中和抗体受動免疫により示されている[21-25]. 完全な感染阻止の可能性については疑問があるものの, 次項(3B)のHIV曝露後の2次反応との協調によりHIV持続感染成立阻止に結びつく機序が示唆されたこともあり, エイズワクチン開発戦略の一つとして, 中和抗体誘導は重要と考えられている. また, 中和抗体産生ウイルスベクター導入サルにおけるSIVチャレンジ実験も(用いられたSIV株の問題はあるものの), 中和抗体誘導の有効性を支持している[26].

中和抗体誘導のための試みとしてこれまで進められた不活化ウイルスワクチンやEnvサブユニットワクチン等の研究は失敗に終わってきた. 特に組換えEnv表面蛋白(gp120)サブユニットワクチンは, 初めての臨床試験第3相まで行われたが, 中和抗体誘導効果もなく有効性を示すことができなかった[27-30]. HIVの中でも培養実験等で用いられている実験室株に対する中和抗体誘導は比較的容易であるが, HIV感染者から得られる臨床分離株に対する中和抗体誘導は困難とされている. 近年の研究進展により, いくつかの臨床分離株に対する中和抗体誘導が可能となってきているものの, HIVの多様性に対応しうるブロード(広範)な中和抗体誘導が難しく, その課題克服が主要なテーマとなっている[31]. 現在, 大規模に進められている戦略は, HIV感染者に時折認められるブロード中和抗体の認識部位(エピトープ)を同定し, 分子構造解析を基にした抗原デザインを試みるものである[1]. これまで, エピトープが同定されたものとしては, gp120のCD4結合部位を認識するb12や高マンノース型オリゴ糖鎖を認識する2G12などのHIV Envのレセプター結合を阻害するモノクローナル中和抗体[32-34], およびEnv膜貫通蛋白(gp41)の膜貫通ドメイン近傍領域を認識する2F5, 4E10などのHIV細胞侵入時の膜融合過程を阻害するモノクローナル中和抗体がある[35]. さらに, 三量体gp120のVループを認識するブロードなモノクローナル中和抗体PG9, PG16が報告され注目されている[36].

3B. ワクチン誘導メモリーBリンパ球反応によるHIV複製抑制の可能性

ここでは, HIV感染成立後の中和抗体反応がHIV複製に与える影響を中心に述べることとする. 前述のように, サルエイズモデルにおけるウイルス感染後の中和抗体受動免疫実験から, 慢性持続感染が成立してしまうと, その後に中和抗体が誘導(あるいは投与)されても, HIV複製制御には至るような効果を示さないと考えられている[2,3]. しかし, 我々のSIV感染急性期(SIV mac239感染7日目)の中和抗体受動免疫実験では, 感染後約1カ月以降の体内ウイルス量の低下が認められ, HIV感染急性期の中和抗体反応がHIV持続感染成立阻止に結びつく可能性が示された[37]. この系では, 抗体を介する樹状細胞へのウイルス粒子取込み亢進による機能的SIV特異的CD4陽性Tリンパ球反応の促進(ならびに中和抗体による保護)という機序が考えられ, 液性免疫と細胞性免疫との協調作用によるHIV複製抑制という新しい概念が示唆された[38,39]. したがって, 中和抗体誘導ワクチンでは, 高力価の中和抗体の誘導・維持ができず[40] HIV曝露時の中和抗体による感染防御が不完全であっても, 曝露後の抗体反応を介した機能的細胞性

免疫反応により，持続感染成立阻止に結びつく可能性が期待される．なお，HIV曝露後のBリンパ球反応などの影響について，サルエイズモデルにおけるBリンパ球枯渇実験の報告[41,42]や，抗体依存性細胞傷害反応（ADCC）に関する報告[43]などがあるが，未だコンセンサスは得られておらず議論が分かれている状況である．

3C. ワクチン誘導エフェクターメモリーCTL反応によるHIV複製抑制の可能性

CTL誘導ワクチンは，抗体誘導ワクチンと同様エフェクターを誘導すると誤解されている場合もあるが，基本的にはメモリーTリンパ球（CTL）を誘導するワクチンである．しかし，メモリーTリンパ球について，エフェクターメモリー（EM）とセントラルメモリー（CM）に大別することができ，HIV曝露後の両者の影響には大きな違いがある．最近の複製型サイトメガロウイルス（CMV）ベクターを用いたワクチンのサルエイズモデルにおける解析結果[44]は，EM-CTL誘導型ワクチンの可能性を示すとともに，EM誘導型とCM誘導型の違いを示唆したもので興味深い．ヒトCMV感染においては，CMV特異的EM-Tリンパ球が誘導・維持されることが知られていたが，HIV抗原発現CMVベクター接種サルでは，効率よくHIV抗原特異的EM-Tリンパ球が誘導・維持され，数頭のサルにおいてSIV複製防御効果が認められた．この防御効果は，感染急性期ピークウイルス量の低下（あるいは検出可能レベルの感染の阻止）が基本で，急性期に有効性が認められなかったものは，セットポイント期のウイルス量の低下もみられないという結果であった．これが，HIV曝露時あるいは曝露直後の迅速なEM-CTL反応によるHIV複製抑制効果の特徴と考えられる．このようにEM-CTLを維持することについては，HIV感染慢性期の過剰な免疫活性化と同様，免疫系の破綻に結びつく可能性も含め，安全性の面ではまだまだ検討すべきことがあるが，ワクチンによるHIV感染・複製阻止に向けた新たな機序の一つとして，今後の研究進展が期待される．

なお，この系と関連して，弱毒化生ワクチンについて述べておく必要がある．サルエイズモデルにおいて，nef遺伝子を欠損したSIVを弱毒化ウイルスとして用い，その有効性を示したものが代表的な報告である[45,46]．これらの研究より，弱毒化生ワクチンの有効性はコンセンサスとなっているものの，慢性持続感染を呈するHIVの弱毒化により発症しない安全な生ワクチンを獲得する原理は確立されておらず[47]，現時点では弱毒化生ワクチンの臨床応用は考えられていない．しかし，弱毒化生ワクチンの野生型SIV持続感染成立阻止の機序を明らかにすることは，重要な課題の一つとされている．中和抗体の関与は無くとも有効性を示すことは報告されている[48]一方，CTLの関与は指摘されているが[49]，それ以外の機序の可能性も検討されている．我々は，上記CMVベクターワクチンと同様，EM CTL誘導が中心的役割を果たしているのではないかと考えている．

3D. ワクチン誘導セントラルメモリーCTL反応によるHIV複製抑制の可能性

我々は，CM-CTL誘導は，CTL誘導ワクチンの基本となる概念と考えている．この系は，HIV曝露後のCM-Tリンパ球由来のCTL反応のHIV複製抑制効果により，セットポイント期の体内ウイルス量低下・持続感染成立阻止を期待するものであり，実際，サルエイズモデルで部分的ながらもワクチン効果が認められた最初の系である（後述のアデノウイルス[AdV]ベクターワクチン[50]およびセンダイウイルス[SeV]ベクターワクチン[51]）．CM-CTL誘導を誘導しておくことのメリットは，より迅速かつ高レベルのCTL反応の誘導が期待されることだけでなく，より機能的なCTL反応の誘導効果や，誘導されるCTLの優位性（ドミナンスパターン）の変化などが考えられる[52]．

このCTL誘導ワクチン開発においては，デリバリーシステムと発現抗原の選択が重要となる．デリバリーシステムとしては，HIV抗原発現ウイルスベクターの有用性が示されており，現時点では，DNAワクチンを組み合わせたDNAプライム・ウイルスベクターブースト法が，最も効率よいCTL誘導法と考えられている．ポックス系ウイルスベクター，AdVベクター，SeVベクターなど各種ウイルスベクターの開発が進められきたが，特にAdVベクターあるいはSeVベクターを用いたプライム・ブーストワクチンについては，部分的ながらもサルエイズモデルにおける有効性（ウイルス量低下ならびに感染急性期のメモリーCD4陽性Tリンパ球の維持）を初めて示した有数のワクチンシステムとして期待されている[12,50,51,53-55]．これらのシステムで誘導される抗原特異的Tリンパ球について，CMVベクター以外は大部分，CM-Tリンパ球誘導・維持を主とするが，さらにその中のサブセットについても検討が進められている．また，複製型あるいは非複製型の選択，接種経路の選択，粘膜免疫誘導の可能性，抗ベクター既存免疫の影響などの検討すべき課題が残っている状況である[56]．

発現抗原の選択は，さらに重要な課題である．この際，どのような抗原エピトープ特異的CTLが強いHIV複製抑制能を有するかという点を明らかにすることが重要である．培養細胞レベルでのCTLのHIV複製抑制能の解析[52,57,58]や，個体レベルでのCTLエスケープ変異の解析，さらにはその変異を有するHIVの複製能の解析[51,53,59-64]は，有効なCTLの選択に有用と考えられる．この点については，サイトカイン産生能などのCTLの機能だけではなく，抗原提示側（標的感染細胞）の抗原提示効率の影響も大きいことに留意する必要がある．さらにHIV多様性への対応も含め，いかに数多くのエピトープ特異的CTLを誘導するかという点も重要な検討課題ではあるが，ウイルス側の多様性

だけでなく宿主側（MHC-I）の多様性の影響をうけることや，ドミナンスパターンの問題などをふまえ，総合的に考えることが重要である．

なお，機能的HIV特異的CTL誘導に必要とされるHIV特異的CD4陽性ヘルパーTリンパ球については，ワクチンによる誘導が，HIV感染標的の増幅を介してHIV複製を促進する可能性を考慮する必要がある[65]．例えば，SIV Env特異的メモリーCD4陽性Tリンパ球誘導が，SIV曝露後のSIV増殖促進に結びつくことを示唆した報告もある[66]．この問題点を克服する一つの戦略として，我々は非HIV抗原特異的CD4陽性ヘルパーTリンパ球とHIV特異的CTLを誘導するワクチンシステムの有効性を提唱している[52]．サルエイズモデルにおける解析では，SIV特異的CD4陽性Tリンパ球のヘルパー反応を誘導しないワクチンによっても，非SIV抗原特異的CD4陽性Tリンパ球とともにSIV特異的メモリーCTLを誘導することで，SIV曝露後に有効なCTL反応が生じうることが示されている．

4. エイズワクチン臨床試験について

予防エイズワクチンの臨床試験のためには，流行地域での大規模な有効性の検証（プラセボとの感染者頻度の比較）が必要となる．その困難さを克服する目的で1990年代後半に設立された国際エイズ推進構想（International AIDS Vaccine Initiative [IAVI]; http://www.iavi.org）は，現在，エイズワクチン臨床試験推進において中心的役割を果たしている．中和抗体誘導ワクチンおよびTリンパ球誘導（ウイルスベクター）ワクチン双方の推進を進めているが，我々がディナベック社と共同で開発を進めてきたSeVベクターエイズワクチンも，IAVIとの国際共同臨床試験計画（米国での第1相）が進展している．サルエイズモデルにおける有効性は部分的ではあるが，ワクチン接種者でHIV曝露後に感染が成立してしまっても体内ウイルス量に低下がみられれば，さらなる伝播の確率を下げることが期待される．実際，体内ウイルス量が1.7×10^3コピー/ml以下であれば感染伝播の確率は大きく減少することが報告されており[67]，我々は第一世代エイズワクチンとして，集団としての感染拡大抑制に結びつく可能性を期待している．

なお，近年の代表的な臨床試験として，メルク社のHIV Gag・Pol・Nef発現AdV血清型5（Ad5）ベクターの有効性試験では，有効性が認められなかっただけではなく，Ad5抗体陽性者の場合，ワクチン接種群の方がHIV感染者の頻度が高くなるという結果が報告された[68,69]．その機序は未だ不明であるが，そのため，サルエイズモデルで部分的ながらも有効性を示したDNAプライム・AdVブーストワクチンの有効性試験も延期される結果となった．近年，ヒトで抗体陽性者の少ない別の血清亜型のAdVベクターワクチン開発が進展している[70]．

一方，タイで行われていたHIV Gag・Pol・Env発現トリポックス（カナリア痘ALVAC）ウイルスベクターとgp120サブユニットワクチンを組み合わせたプライム・ブーストワクチン臨床試験第3相にて，HIV感染者頻度に若干（約30%）の有効性が認められたとの報告が2009年9月になされた[71]．サルエイズモデルにおいてSIV複製抑制効果が認められなかった系であり[72]，今回の臨床試験でも，プロトコール通りワクチン接種を受けた者同士のプラセボとの比較では有意差が無く，またハイリスク群でも効果が認められない微妙なものであるが，今後の免疫学的解析結果の発表が待たれるところである．

5. まとめ

予防エイズワクチン研究においては，ワクチンにより誘導すべき免疫機序を明らかにすることが肝要である．近年の研究により，中和抗体誘導ワクチンについては，HIV曝露時の抗体によるHIV感染防御とHIV曝露後の液性免疫と細胞性免疫の協調反応によって持続感染成立阻止に至る機序が示された．CTL誘導ワクチンについては，HIV曝露後のCM-CTL反応によるHIV複製抑制に加えて，EM-CTL反応によるHIV複製抑制の可能性も指摘されている．このような機序のさらなる解明が，持続感染成立阻止効果を有する第二世代エイズワクチン開発に結びつくことを期待している．

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Theory for prophylactic AIDS vaccine development

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In the natural courses of human immunodeficiency virus (HIV) infections, host immune responses fail to contain the virus and allow persistent HIV replication, leading to AIDS progression. For development of an effective vaccine against those viral infections which do not show spontaneous remission, it is important to elucidate which immune responses to be induced for viral control. This review focuses on antibodies and cytotoxic T lymphocytes, key adaptive immune effectors, and discusses possible mechanisms for HIV control by vaccine-induced antibody, memory B lymphocyte, and (effector and central) memory T lymphocyte responses. Finally, we mention the ongoing international project for a clinical trial of our Sendai virus vector-based AIDS vaccine.

IL-4/IL-13 antagonist DNA vaccination successfully suppresses Th2 type chronic dermatitis

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Summary

Background Atopic dermatitis (AD) is a chronic disease with a Th2-type-cytokine dominant profile. Several cytokines and related peptides have been used for the treatment of AD but they were ineffective because of their limited biological half-life. We have recently developed a highly efficient mouse dominant negative interleukin (IL)-4/IL-13 antagonist (IL-4DM), which blocks both IL-4 and IL-13 signal transductions.

Objective To examine the effects of IL-4DM in vivo in an AD model induced by the repeated exhibition of oxazolone (OX).

Methods Plasmid DNA was injected intraperitoneally to cause an experimental AD-like dermatitis. The effect was evaluated by ear thickness, histological findings, and mast cells counts in the inflamed skin. The plasma IgE and histamine levels were measured. Cytokine production in skin and splenocytes were also analysed.

Results Mice treated with control plasmid developed marked dermatitis with mast cells and eosinophil infiltration, and had increased plasma IgE and histamine levels with a Th2 type splenocyte cytokine profile. Treatment with mouse IL-4 DNA augmented the ear swelling and thickness with an increased dermal eosinophil count, plasma histamine level, and production of splenocyte IL-4. However, IL-4DM treatment successfully controlled the dermatitis, decreased the mast cell and eosinophil count, and suppressed plasma IgE and histamine levels. Splenocytes produced an increased level of IFN-γ.

Conclusion These data showed that the simultaneous suppression of IL-4/IL-13 signals successfully controlled Th2-type chronic dermatitis. IL-4DM DNA treatment is a potent therapy for AD and related diseases.

Interleukin (IL)-4 plays a central role in Th2-cytokine-dominant inflammatory skin diseases such as atopic dermatitis (AD).[1–3] IL-4 is responsible for the differentiation of allergen-specific Th2 cells together with its closely related cytokine IL-13 for the class switching of activated B cells to IgE-producing cells. The effects of IL-13 are similar to IL-4 on B cells, monocytes, and other cell types, but T cells appear to lack an IL-13 binding receptor component and do not respond to IL-13.[4] The structural basis for the overlapping functions of IL-4 and IL-13 is a shared receptor subunit, and IL-4Rα organizes intracellular signals in response to both cytokines.[5,6] Signal transduction is induced by heterodimerization of the IL-4Rα with a second subunit; which may vary according to the cell types. The specific inhibition of IL-4 can be achieved by antagonistic IL-4 mutants. Variants of human IL-4 that bind to the receptor subunit IL-4Rα, but not to the other subunit γ-chain (γc) or IL-13Rα1 are competitive antagonists of IL-4.[7,8] IL-13 is inhibited by similar variants, which form unproductive complexes with IL-4Rα.[5,9] The single-site human IL-4 mutant Y124D has been used as an IL-4/IL-13 inhibitor in various studies,[7–17] but this variant retains some residual agonistic activity, which could be relevant for in vivo applications.[7,8] In contrast, IL-4 and IL-13 double mutant R121D/Y124D lacks detectable activity and appears to be an effective antagonist for human IL-4 and IL-13.[5,18]

We have recently developed a highly efficient murine IL-4 antagonist DNA (IL-4DM), in which the amino acids glutamine 116 and tyrosine 119 were changed for aspartic acid.[19] This murine mutant DNA is analogous to the R121D/Y124D double mutant. IL-4DM binds with high affinity to the murine

IL-4Rα without inducing signal transduction, and has no detectable activity upon the proliferation or differentiation of murine cells. An appropriate amount of IL-4DM completely inhibits responses by wild-type IL-4.[19] Like its human analogue, the IL-4DM mutant is also an antagonist of IL-13 (B. Schnarr *et al.*, unpublished data[37]). Recent experiments with monocytes from mice lacking a functional γc gene showed that IL-4DM is a complete inhibitor of IL-4 in the absence of γc as well.[20] In this study we have examined the effects of IL-4DM *in vivo*, using an AD model induced by the repeated exhibition of oxazolone (OX). The repeated application of a hapten such as OX on mice causes an initial delayed-type hypersensitivity that changes to an immediate-type response in the late phase with elevated IgE production and deviation of Th-cell responses. The skin lesions that appear in the late phase are compatible with the clinical findings as well as the cytokine profile observed in AD.[21–23] The inhibitory effect of IL-4DM on IL-4 and IL-13 on the immune response was comparable with that of knockout mice lacking either IL-4[24] or IL-4Rα. Treatment with IL-4DM prevented contact hypersensitivity responses with the increased production of interferon (IFN)-γ.

Materials and methods

Animals

BALB/c male mice aged 5 weeks were purchased from Japan SLC Co. (Shizuoka, Japan) and were used at the age of 6 weeks. Age-matched wild-type BALB/c mice were used as controls. All animals were cared for according to the ethical guidelines approved by the Institutional Animal Care and Use Committee of Mie University.

Reagents

The cDNA coding region of mouse IL-4 was amplified by a polymerase chain reaction (PCR) based on the cDNA sequence of mouse IL-4. The mouse IL-4 fragment was inserted into BamHI and EcoRI-filled in pcDNA3.1+ (Invitrogen, San Diego, CA, U.S.A.) under the TPA leader sequence, and then digested by BamHI and SacI. A Quickchange™ Site-directed Mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) was used for the mutagenesis of mouse IL-4. The oligonucleotide primers used to prepare a mouse IL-4 double mutant (IL-4DM, Q116D/Y119D) were CTAAAGAGCATCATGGATATGGATGACTCGTAGTCTAGAG and CTCTAGACTACGAGTCATCCATATCCATGATGCTCTTTAG. The IL-4 mutant fragments were ligated into pcDNA3.1+.[25] Mouse IL-4, IL-4DM plasmid DNAs were purified using the Plasmid Mega kit (Qiagen, Chatsworth, CA, U.S.A.) and diluted with sterilized physiological saline. OX was purchased from Sigma (St Louis, MO, U.S.A.) and was dissolved in acetone/olive oil (4 : 1).

Administration of DNA

Mice were treated by intraperitoneal injection of 100 μg of IL-4DM DNA on days 0, 7, 14, 21 and 28. A control plasmid (pcDNA3.1+) vector and IL-4 DNA were also injected on the same day (Fig. 1).

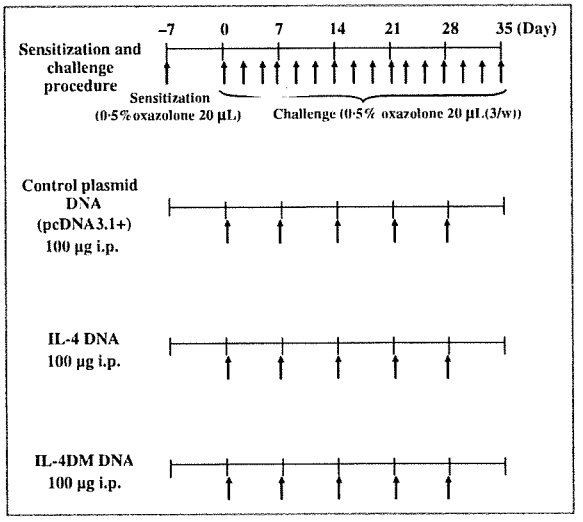

Fig 1. Schedule for induction of chronic contact hypersensitivity and administration of compounds. Mice received intraperitoneal (i.p.) injection of 100 μg of each plasmid DNA on days 0, 7, 14, 21 and 28.

Sensitization and challenge procedures

As shown in Figure 1, mice were initially sensitized by pasting 20 μL of 0·5% OX solution to their left ear 7 days before the first challenge (day 7) and then 20 μL of 0·5% OX solution was repeatedly applied on the left ear three times per week from day 0 as reported previously.[23] Ear swelling was measured with thickness gauge calipers before and 30 min after OX challenge to the pinna of the ear on day 35. The ear swelling response was expressed as the difference between the values taken before and 30 min after application.

Histological analysis

Ear skin specimens obtained 6 h after the final challenge on day 35 were fixed in 10% buffered neutral formaldehyde and embedded in paraffin wax. Histological sections were of 6 μm thickness and they were stained with haematoxylin and eosin. The sections were also stained with 0·5% toluidine blue for the identification of mast cells. The cell counts were performed in six consecutive microscopic fields at × 400 magnification.

Measurement of plasma IgE and plasma histamine

Blood was collected under ether anaesthesia 6 h after the last challenge. Plasma IgE levels were determined by a sandwich enzyme-linked immunosorbent assay (ELISA). In brief, 96-well immunoplates (Corning Inc., Corning, NY, U.S.A.) were coated with 100 μL of an antimouse IgE capture antibody (2 μg mL^{-1}) (BD PharMingen, San Diego, CA, U.S.A.) overnight at 4 °C. Plasma samples of 100 μL were diluted 60-fold with PBS

containing 10% fetal calf serum (FCS) were placed in the wells. After incubation for 1 h at room temperature, 100 μL of biotin-conjugated antimouse IgE antibody (2 μg mL^{-1} in blocking buffer) (BD PharMingen) was added to each well. The plates were incubated at room temperature for 1 h, followed by six washes, incubated with 100 μL of horseradish peroxidase avidin D (FUNAKOSHI, Tokyo, Japan) 1 : 1000 in blocking buffer, and then incubated for 30 min at room temperature. A substrate solution of 100 μL containing 1·5 mg ABTS (Sigma-Aldrich, St Louis, MO, U.S.A.) in 5 mL of a 0·1 mol L^{-1} citric acid solution was added, and kept for 30 min at room temperature in a dark place. Thereafter the reaction was terminated by adding 50 μL of 2 mol L^{-1} H_2SO_4, and the optical density of each well at 405 nm was determined by using a microplate reader. A standard curve was prepared using mouse anti-TNP IgE standard (BD PharMingen). Plasma histamine levels were analysed using the commercial sandwich ELISA kit from Immunoteck (Marseille, France) according to the manufacturer's protocol.

Purification of mRNA from mouse ears

At 6 h after the final challenge, the skin of the left ear was sampled. The specimen was homogenized and the total RNA was extracted using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instruction; 1 mL of homogenate was vigorously mixed with 200 μL of chloroform, and centrifuged at 12 000 *g* for 15 min at 4 °C. The aqueous phase was separated and mixed with 0·5 mL of 2-propanol (Nacalai Tesque, Kyoto, Japan) to precipitate RNA. After centrifugation, the precipitate was washed with 1 mL of 75% ethanol (Nacalai Tesque) and dried. RNA was suspended in 50 μL of RNase-free water, and the concentration was measured based on the absorbance at 260 nm, and the quality was confirmed by electrophoresis. cDNA was prepared from 10 μg of mRNA using archive kit (ABI, Foster City, CA, U.S.A.) according to the manufacturer's protocol.

Cytokine mRNA expression in skin

The transcriptional activity in the lesional skin samples was measured with a PCR. The amplification of cDNA was performed in 50 μL of a master mixture containing 0·5 μg of cDNA, 200 nmol deoxynucleotide triphosphate, 5 μL of PCR buffer, 2 U of Taq polymerase (ABI) and 2 μmol of each specific primer for the DNA of interest. The following primers were used for PCR reactions (5′–3′), mouse IFN-γ: TCAAGTGGCATAGATGTGGAAGAA and TGGCTCTGCAGGATTTTCATG; mouse IL-2: CCTGAGCAGGATGGAGAATAACA and TCCAGAACATGCCGCAGAG; mouse IL-4: CACTGACGGCACAGAGCTATTGATG and TCATGGTGCAGCTTTCGATGAATC; mouse IL-10: CTCTTACTGACTGGCATGAGGATCAGCAGG and TCTTCACCTGCTCCACTGCCTTGCTCTTAT; mouse IL-12: TCCTGCACTGCTGAAGACATC and TCTCGCCATTATAGATTCAGAGAC; mouse IL-13: AGACCAGACTCCCCTGTGCA and TGGGTCCTGTAGATGGCATTG; mouse β-actin: TGGAATCCTGTGGCATCCATGAAAC and TAAAACGCAGCTCAGTAACAGTCCG.[26] PCR was performed under the following conditions: 95 °C for 5 min, followed by 35 or 40 cycles of 95 °C for 30 s, 56 °C (IFN-γ, IL-12) or 60 °C (IL-2, IL-4, IL-10, IL-13, β-actin) for 30 s, and 72 °C for 1 min were carried out. After the final cycle, the temperature was maintained at 72 °C for 7 min. PCR amplified fragments were electrophoresed through 1·5% agarose gels in tris-acetate EDTA buffer containing ethidium bromide, and the gels were scanned under ultraviolet light. The mRNA of β-actin was used as an internal control. The signal intensity of each reverse transcriptase (RT)-PCR product was estimated using an ATTO Lane & Spot Analyzer (ATTO, Shizuoka, Japan).

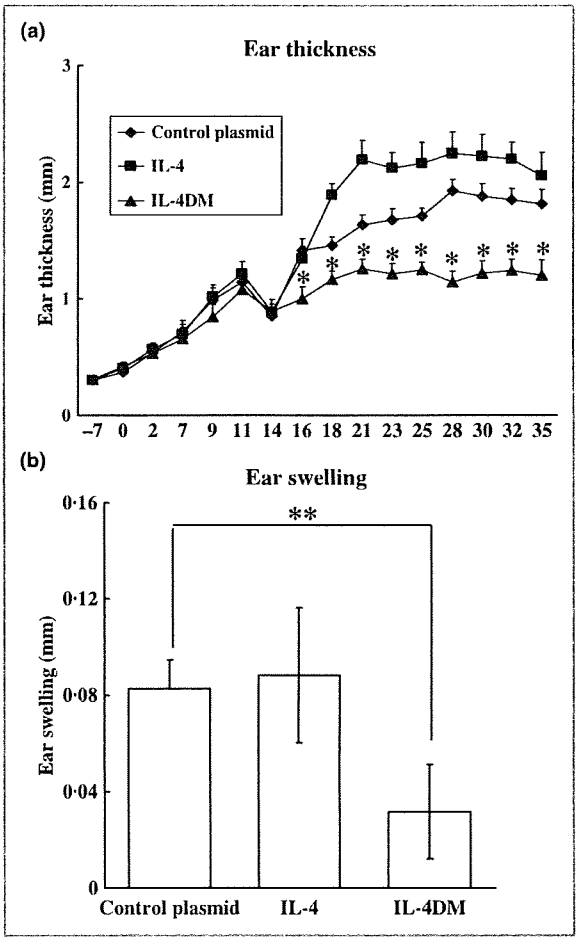

Fig 2. The effects of interleukin (IL)-4DM, IL-4, and control plasmid (pcDNA3.1) on ear swelling induced by repeated application of oxazolone (OX). (a) Ear thickness was measured before each OX challenge. Each point represents the mean ± SD of seven or eight mice. *$P < 0·05$: significantly different from the control group and IL-4 (Student's t-test). (b) Inhibition of the effecter phase of chronic hypersensitivity by IL-4DM, IL-4, and control plasmid DNA transfer. The ear swelling was measured 30 min after applying OX. The ear swelling in the IL-4DM groups was significantly suppressed compared with those in the IL-4 and control plasmid DNA groups. *Significant difference from the control by Student's t-test at $P < 0·05$.

Cytokine production from splenocytes

A suspension of 2×10^6 splenocytes were made in a solution of 200 μL RPMI-1640 medium (Nikken Bio Medical Laboratory, Kyoto, Japan) containing 10% fetal bovine serum (FBS; Biowest, Nuaillé, France), 50 UI penicillin, 50 μg mL^{-1} streptomycin, and 5 μg mL^{-1} soluble antimouse CD3 (BD Bioscience), and 10 μg mL^{-1} antimouse CD28 (BD Bioscience). Cells were dispensed in triplicate into 96-well flat-bottomed microplates (Sumitomo Bakelite, Tokyo, Japan). After incubation for 48 h at 37 °C in a humidified incubator (5% CO_2), culture supernatants were collected and analysed for IFN-γ (Quantikine; R&D Systems, Minneapolis, MN, U.S.A.) or IL-4 (Quantikine; R&D Systems) production with an ELISA according to the manufacturer's protocol.

Statistical analysis

Statistical analysis was performed using Student's t-test and Mann–Whitney U-test. Values are expressed as mean ± SEM. A 95% confidence limit was taken as significant ($P < 0.05$).

Result

Ear thickness with the treatment of IL-4DM, IL-4, or control plasmid

In the control group, the ear thickness increased from the beginning of the challenge, and increased gradually through the experiments (Fig. 2a). The agonistic IL-4 DNA treatment augmented increase of the ear thickness after day 16. In contrast, IL-4DM DNA treatment significantly suppressed increase of the ear thickness compared with that of control plasmid or IL-4DNA-treated mice.

Effects of IL-4DM on the oxazolone-induced acute-phase ear swelling

The ear swelling was also measured 30 min after OX application on day 35, and the difference between before and 30 min after application was calculated. IL-4DM DNA treatment suppressed the ear swelling significantly compared with that of the control DNA-injected group (Fig. 2b). However, IL-4DNA showed no suppressive effects.

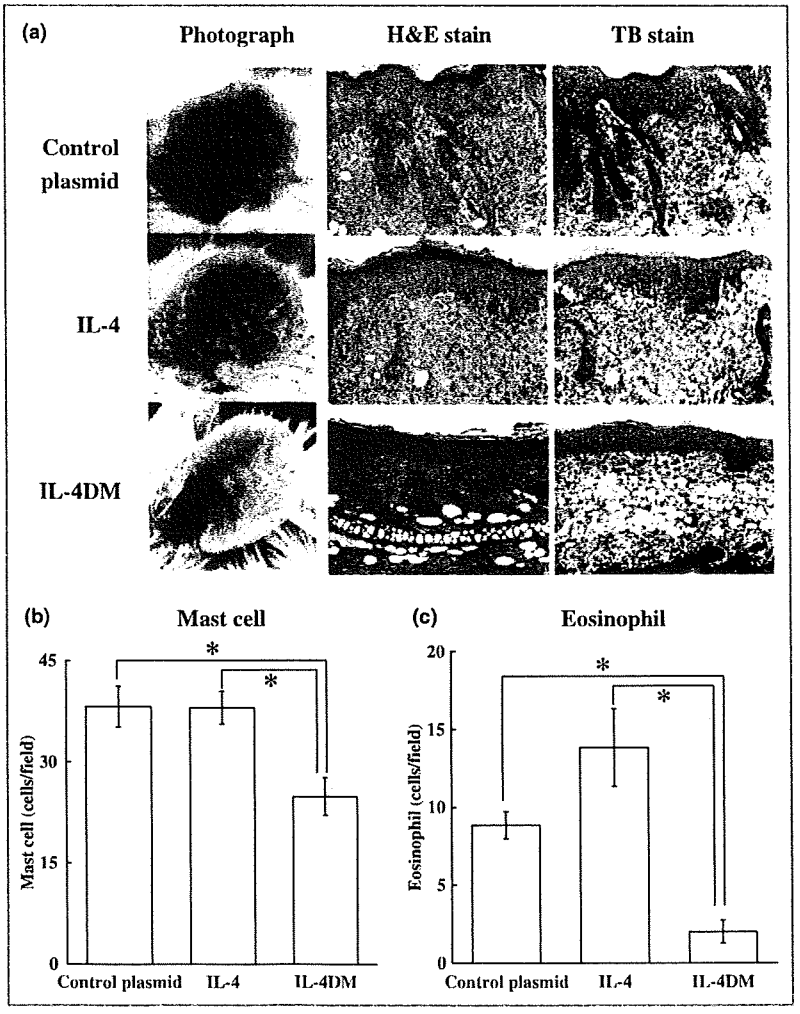

Fig 3. (a) Representative photographs and histological feature of oxazolone (OX)-treated skin lesion. OX-sensitized ear revealed hyperkeratosis, acanthosis, and parakeratosis in control and interleukin (IL)-4-treated mice. An increased number of infiltrating lymphocytes, macrophages and mast cells was observed in the skin lesions, all of which are typical histological findings observed in patients with atopic dermatitis. In contrast, acanthosis was clearly suppressed, and skin infiltration of granulocytes, eosinophils, and mast cells was decreased in the IL-4DM-treated mice as compared with control plasmid-treated mice (original magnification × 200). (b) The number of dermal mast cells was counted, and found to be decreased in the IL-4DM-treated mice. (c) The number of dermal eosinophils was also counted in 10 high power fields. The skin infiltration of eosinophils was significantly decreased in the IL-4DM-treated mice. Data are expressed as the mean ± SEM. *Significant difference by Student's t-test at $P < 0.05$.

Histological findings and mast cell counts in the inflamed skin

In control plasmid-treated mice and IL-4 DNA-treated mice, severe dermatitis was observed on the earlobe. A drastic decrease of inflammation was observed in IL-4DM DNA-treated mice (Fig. 3a). Histological examination on the OX-challenged ear skin revealed hyperkeratosis, acanthosis and parakeratosis in both of the control and IL-4-treated mice. An increased number of infiltrating lymphocytes, macrophages and mast cells was observed in the skin lesions in control DNA and IL-4 DNA-treated mice. These findings are comparable with those of AD skin lesions. In contrast, the acanthotic changes and infiltration of granulocytes, mast cells, and eosinophils were significantly suppressed in the IL-4DM DNA-treated mice compared with those of control DNA- or IL-4 DNA-treated mice (Fig. 3b,c). Interestingly, IL-4 DNA treatment increased eosinophil counts compared with control DNA treatment.

Plasma IgE and histamine levels

The total plasma IgE level was increased by repeated OX challenges (Fig. 4a). IL-4 DNA treatment showed no agonistic effects in the plasma IgE level; however, IL-4DM DNA treatment significantly suppressed the levels of plasma IgE. The plasma histamine level was also significantly increased in the control DNA- or IL-4 DNA-treated mice; however, IL-4DM DNA treatment significantly suppressed the plasma histamine levels (Fig. 4b).

Cytokine mRNA expression levels

To determine the effects of IL-4DM on cytokine production in the inflamed skin lesions, mRNA expression of Th1 and Th2 cytokines was analysed. The IFN-γ mRNA expression was significantly increased in IL-4DM DNA-treated mice ear compared with that of control DNA-treated samples (standardized by β-actin expression).

However, no remarkable difference in other cytokine mRNA expression was observed among three different DNA-treated samples (Fig. 5a,b).

Concentration of IFN-γ and IL-4 in splenocyte cell culture supernatants

To know the effects of IL-4DM DNA therapy in the systemic immune system, the concentration of IFN-γ in splenocyte cell culture supernatants was measured by ELISA. The IFN-γ level in the IL-4DM-treated samples was significantly higher than that in the control DNA- or IL-4 DNA-treated samples (Fig. 6a). We also measured the concentration of IL-4 in splenocyte cell culture supernatants by ELISA for mouse IL-4. The IL-4 level in the IL-4DM-treated samples was as high as the IL-4 DNA-treated samples. These were higher than that of the control DNA-treated samples (Fig. 6b).

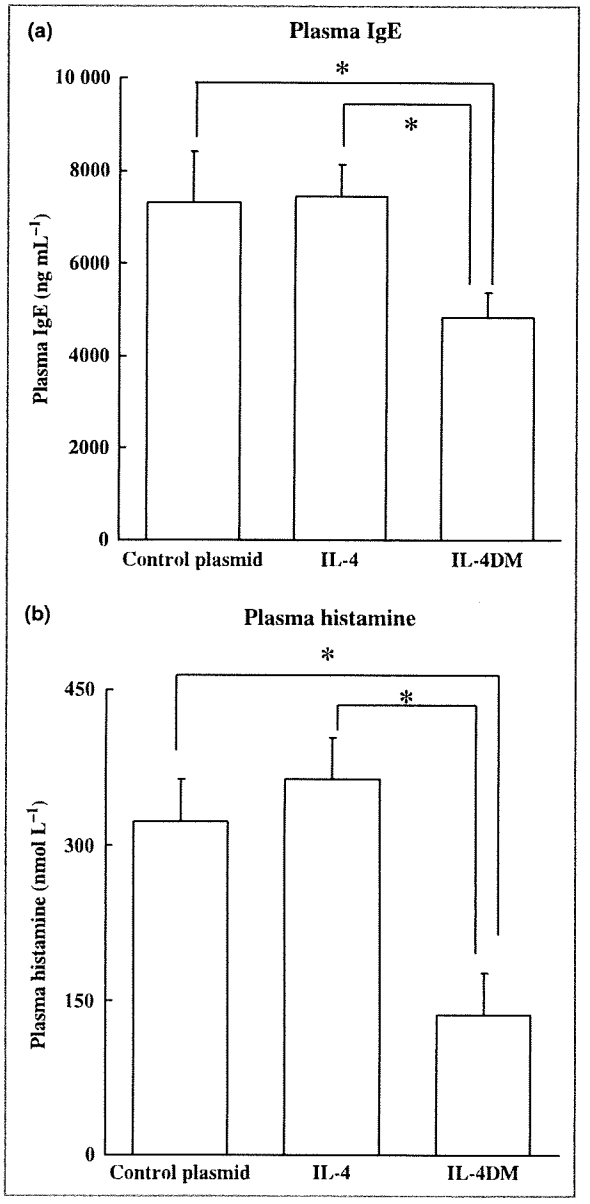

Fig 4. Plasma IgE and histamine levels. (a) Plasma IgE level was decreased in interleukin (IL)-4DM treated mice. (b) Inhibition of the production of plasma histamine was observed in IL-4DM DNA-treated mice. *Significant difference from the IL-4 and control by Mann–Whitney U-test at $P < 0{\cdot}05$.

Discussion

Several previous studies have shown that AD is a chronic dermatitis with a predominance of Th2 cytokines in the lesional skin,[27–29] and that Th2 cytokines play a critical role in the pathogenesis of dermatitis.[28] IL-4 is one of the Th2 cytokines that affects the function of different cell types including T cells, B cells, mast cells, monocytes/macrophages, endothelial cells, fibroblasts, dendritic cells, Langerhans cells

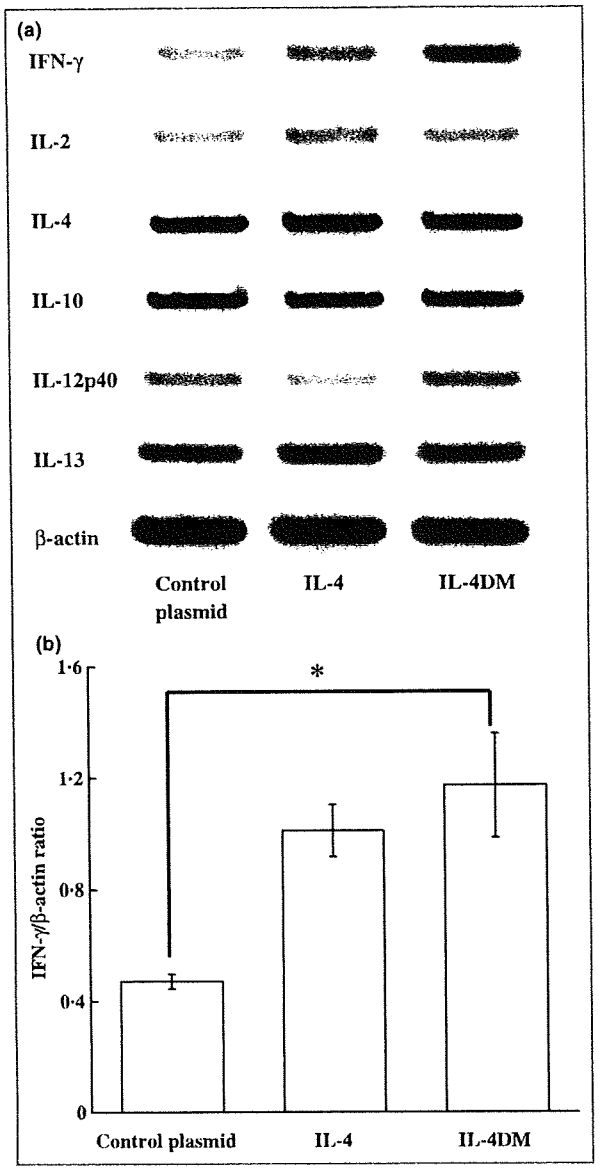

Fig 5. (a) Reverse-transcriptase polymerase chain reaction analysis of cytokine mRNA expression 6 h after oxazolone (OX)-sensitization. The cDNAs were amplified for respective cycles of six cytokines and β-actin, subjected to electrophoresis, and visualized with ethidium bromide. Representative results under optimal conditions are shown. Although almost all Th1 and Th2 cytokine levels were unchanged, mRNA expression for interferon (IFN)-γ was increased in IL-4DM-treated mice. (b) The level of mRNA expression of IFN-γ was expressed as the value relative to that for β-actin. The IFN-γ level in the IL-4DM group is significantly higher than that of control plasmid groups. *Significant difference from the control by Student's t-test at $P < 0{\cdot}05$.

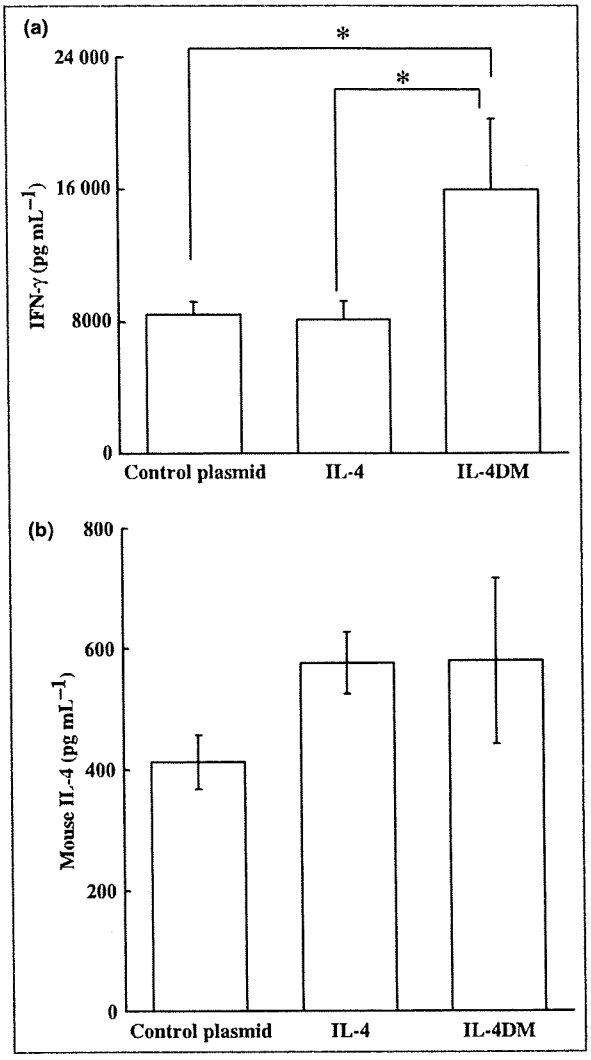

Fig 6. Cytokine production from splenocytes in chronic hypersensitivity mice. Interferon (IFN)-γ and interleukin (IL)-4 production from splenocytes was measured. (a) Actual IFN-γ protein production was increased in the IL-4DM-treated mice. (b) IL-4 levels did not reach the significance, but showed a tendency to increase in the IL-4 and IL-4DM mice. *Significant difference from the control by Mann–Whitney U-test at $P < 0{\cdot}05$.

and keratinocytes. Because of this broad-spectrum action, IL-4 is believed to play a crucial role in the pathogenesis of AD.[30,31] In the present study, we employed a contact hypersensitivity model by the repeated application of OX, which mimics the histological phenotype of AD in humans; this model also showed increased levels of Th2 cytokines in the lesional skin as reported by Kitagaki et al.[21]

Immunotherapy such as the direct blocking of Th2 responses with neutralizing antibody against Th2 cytokines, the soluble form of IL-4 receptor (IL-4R), or antagonistic IL-4 mutant proteins have been used for the treatment of asthma.[32–34] These proteins directly inhibit IL-4 binding thereby inhibiting host immune responses. A previous study by Nishikubo et al.[25] showed inhibition of immune responses by using IL-4 mutant protein for at least 50 weeks. However, results from these experimental animals have shown that the application of these trials to humans is difficult. Because the pharmacokinetic half-life of IL-4 mutant and sIL-4R protein

