Promiscuous Interaction between Gold-Specific T Cells and APCs in Gold Allergy

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Gold compounds have been widely used in the treatment of autoimmune diseases such as rheumatoid arthritis (RA) to abrogate the immune functions of autoreactive T cells. Although the exact mechanisms of their beneficial actions have never been fully explored, gold compounds inhibit NF-κB and IFN regulatory factor nuclear translocation and expression of cyclooxygenase-2 to prevent immune cells from becoming activated and producing cytokines in vitro. Gold also abrogates Ag presentation by binding to MHC-peptide complexes to alter their conformation (2) and/or stripping peptides from MHC molecules (3), thereby shutting off T cell activation. In contrast, hypersensitivity reactions, including interstitial pneumonitis, hepatitis, and rashes involving the mucosa and skin, occasionally occur in association with gold treatment (4, 5). Modification of MHC molecules with gold compounds may be one explanation for increasing the chances of generating new Ags that provide a high potential for eliciting adverse immune responses (6).

The classical view of immune recognition of hapten by T cells predicts that drugs/metals bound to large molecules such as cell membrane proteins and/or MHC-peptide complexes are presented by APCs with and without internal processing (6–10). In this scenario, new Ags are generated by covalent binding of these hapten to self-derived proteins. The other intriguing implication is the formation of drug/metal-TCR-MHC complexes via reversible noncovalent binding that is sufficient to elicit T cell activation (7). In this setting, drugs and metals may directly cross-link TCRs to MHC molecules, independently of the nature of the associated peptide. Therefore, drugs and metals would act as nonclassical hapten.

A previous study indicated that gold binds directly to the HLA-DR1-peptide complex without Ag processing by APCs to induce T cell activation in gold hypersensitivity (2), although the precise mechanism remains to be elucidated. To explore the mechanism of T cell recognition of drugs, we investigated the phenotypes, cytokine production profiles, chemotactic activities, and MHC dependencies of gold-specific T cell clones (TCCs) and T cell lines (TCLs) generated from a patient with RA who was sensitive to gold.

Materials and Methods

Study participants

Three patients with RA (one male aged 62 years, and two females aged 54 and 73 years) diagnosed according to the American Rheumatism Association criteria and five healthy individuals (three males and two females; mean age, 47 years; age range, 38–60 years) were enrolled in this study. The study was performed according to the Helsinki declaration, and the study protocol was approved by the Hamamatsu University School of Medicine Ethical Committee. Written informed consent was obtained from all participants.

The clinical data of the 73-year-old female with RA who developed skin rashes were as follows. Gold sodium thiomalate was administered at a dose of 20 mg weekly for the treatment of joint pain. After 5 wk of therapy, she had a high fever and developed erythema, petechiae, and papules involving the trunk, extremities, face, and oral enanthema (Fig. 1A). The skin histology revealed inflammatory infiltration of neutrophils and lymphocytes in the epidermis and upper dermis (Fig. 1B). Lymphocytes positive for CD4 and CD8 showed comparable degrees of infiltration (Fig. 1C). Immunohistology revealed that T cells expressing Vβ2, Vβ5.1, and Vβ21.3 were concentrated in the lesional skin with a few T cells bearing Vβ8, Vβ11, or Vβ12 (Fig. 1D). After discontinuing the gold therapy, the fever and eruptions resolved at 3 wk after their onset. At 4 wk after the recovery of the skin rashes, patch tests with HAuCl4 were positive, and the proliferative response of PBMCs to gold at a dose of 10 μg/ml was 2.4 (Fig. 1E). Based on these data, a diagnosis of gold-induced eruptions was made. HLA typing was performed by either the Terasaki-National Institutes of Health
standard method in fresh PBMCs or restriction fragment-length polymorphism analysis of genomic DNA from stored PBMCs. The MHC haplotypes of the patient were A26/33, B56/44, Cw7, and DR9.

The other two patients with RA were treated by oral administration of nonsteroidal anti-inflammatory agents and weekly injection of gold sodium thiomalate at a dose of 20 mg/day. Laboratory examinations of these patients revealed mild increases in serum liver enzymes and consistently high C-reactive protein levels. These two patients had never experienced adverse effects in relation to gold treatment. Among the normal individuals, two served for the establishment of TCCs/TCLs and the other three contributed to the blocking study.

Reagents, mAbs, and culture medium
Because the Au(III) metabolite formed in vivo has been reported to be responsible for the allergic side effects of gold agents (3), we used HAuCl₄ (Wako Chemical) throughout this study. mAbs against MHC class I (anti-HLA-A,B,C: G46-2.6), class II (anti-HLA-DR: G46-6 and anti-HLA-DQ, DR), and Vαβ common frame and a panel of 22 FITC-conjugated mAbs recognizing different TCR Vβ gene products were obtained from BD Pharmingen. FITC-conjugated and purified anti-CD3 (SK7), anti-CD4 (SK3), and anti-CD8 (SK1) mAbs were purchased from BD Biosciences. mAbs against CCR1 to CCR9 and CXCR1 to CXCR6; chemokines including IL-8, TARC, and IFN-γ-inducible protein-10 (IP-10); and goat polyclonal Abs against thymus and activation-regulated chemokine (TARC) and IP-10 were obtained from R&D Systems. A goat polyclonal Ab to CCR4 was obtained from Abcam. Cells were cultured in RPMI 1640 medium (Life Technologies) supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), 5 × 10⁻³ M 2-ME, 1% of a 100× mixture of nonessential amino acids (Life Technologies), and 10% heat-inactivated FCS or pooled human AB serum. For expansion of TCCs and TCLs, the medium was supplemented with 25–50 μl/ml human rIL-2 (Takeda Pharmaceutical).

Cell preparation
A blood sample was taken from the RA patient with skin rashes at 2 mo after discontinuation of the gold therapy and heparinization. PBMCs were isolated from the heparinized whole blood samples by Ficoll-Hypaque (Pharmacia Fine Chemicals) gradient centrifugation. Neutrophils were purified from some oligoclonal TCLs by a MACS method after incubation with a FITC-conjugated anti-Vβ mAb, followed by reaction with anti-FITC microbeads (CD3/CD28 T cell expander; Dynal). The T cells thus raised were maintained in medium supplemented with 25–50 U/ml rIL-2. Monoclonal TCCs were purified from some oligoclonal TCLs by a MACS method after incubation with a FITC-conjugated anti-Vβ mAb, followed by reaction with anti-FITC microbeads. Monoclonality or oligoclonality of the generated TCCs and TCLs was analyzed by anti-TCR Vβ chain mAbs by flow cytometry (FCM).

FCM analysis
Aliquots of 1 × 10⁵ cells were stained with FITC-conjugated mAbs and analyzed, as previously described (11).

Cytokine production assay
Established TCCs/TCLs were stimulated with an immobilized anti-CD3 mAb for 72 h to assess the cytokine production. The concentrations of IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IFN-γ, and TNF-α in culture supernatants were measured by the cytometric bead array (CBA) assay (Th1/Th2 cytokine and inflammation cytokine CBA kits; BD Biosciences), according to the manufacturer’s protocols.

Cytotoxicity assay
Cytotoxicity assays were performed with a LIVE/DEAD cell-mediated cytotoxicity kit (Molecular Probes), according to the manufacturer’s protocol. Briefly, after labeling of target cells with DiOC₆(3), effecter cells were cocultured with the target cells at an E:T ratio of 10:1 in the absence or presence of gold concentrations ranging from 0.1 to 50 μg/ml for 12 h. To enumerate dead target cells, cells stained with propidium iodide were analyzed by FCM.

Real-time horizontal chemotaxis assay
An optically accessible horizontal chemotaxis apparatus (EZ-TAXIScan; GE Healthcare) was used to evaluate the chemotactic activities of cells, as

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**FIGURE 1.** Clinical and laboratory findings of a RA patient with skin rashes. A. Maculopapular rashes. B. Pathology of a biopsied skin lesion. H&E staining. C. Immunohistochemical staining. Arrows, positively stained cells. D. Immunohistochemical staining of the skin lesion with anti-Vβ mAbs. Arrows, positively stained cells. E. PBMCs from the patient exhibit significant proliferation in response to gold. Vertical bars, SD.
previously described (12). The apparatus consisted of front and back chambers containing cells and a chemoattractant, respectively, which were connected by a microchannel. Cumulative numbers of cells present within the microchannel (50 μm in length) during 1-min observation periods were successively recorded at 30-s intervals on a computer equipped with a video camera. Data were analyzed using the Image J software (National Institutes of Health) and the Manual Tracking plug-in produced by FP Cordelieres (Institut Curie; http://rsb.info.nih.gov/ij/plugins/manual-tracking.html).

Histology and immunostaining of skin lesions
Punch-biopsy specimens (diameter, 4 mm) from acute erythematous lesions were fixed in 4% formalin and routinely stained with H&E. For immunohistochemistry, deparaffinized specimens were autoclaved in 10 mM citrate buffer (pH 6.0) for 10 min at 120°C to retrieve the antigenic epitopes and analyzed for their CD4, CD8, CCR4, CXCR3, TARC, and IP-10 expression levels. Alternatively, the skin specimens were snap frozen in tissue-embedding medium, cut at 4-μm thickness, and processed for analysis of TCR Vβs. Following incubation with primary mAbs, positively stained cells were detected by the avidin-biotin complex method with a HISTOFINE staining kit (Nichirei Biosciences). Deoxyaminobenzidine was used as a substrate for visualization of positive reactions. Nuclear staining was performed with hematoxylin. In control experiments, the primary Ab was substituted with isotype-matched IgG or omitted.

Statistical analysis
Student’s t test was used for analysis of differences. Values of p < 0.05 were considered to indicate statistical significance.

Table I. Profiles of representative TCCs/TCLsa

<table>
<thead>
<tr>
<th>Clones</th>
<th>Phenotype</th>
<th>Vβb</th>
<th>Stimulation Index</th>
<th>Cytokine Profilec</th>
<th>Blockingd</th>
<th>Cytokine Levels (pg/ml) (IFN-γ/IL-2/IL-4/IL-8)</th>
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<tr>
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<td>13.8</td>
<td>0</td>
<td>I/II</td>
<td>131740/8933/26075/15801</td>
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<tr>
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<td>n.d.</td>
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<td>8.3</td>
<td>1</td>
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<th>Stimulation Index</th>
<th>Cytokine Profilec</th>
<th>Blockingd</th>
<th>Cytokine Levels (pg/ml) (IFN-γ/IL-2/IL-4/IL-8)</th>
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</thead>
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<td>II</td>
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<tr>
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<td>CD4</td>
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<td>0</td>
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<tr>
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<tr>
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<td>1</td>
<td>I</td>
</tr>
<tr>
<td>F11D3</td>
<td>CD8</td>
<td>7/17</td>
<td>4.2</td>
<td>1</td>
<td>I/II</td>
</tr>
<tr>
<td>E10D9</td>
<td>CD8</td>
<td>7/16</td>
<td>2.6</td>
<td>1</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

a Clone/Line names are given.
b Monoclonality and oligoclonality were confirmed by FCM using a battery of mAbs against TCRVβ. ?, Indicates unknown Vβ by this analysis. Underlines indicate shared Vβs.
c Cytokine production profiles are indicated as 1 for type 1 and 2 for type 2.
d Inhibition of T cell proliferation by anti-HLA-A,B,C (class I) and anti-HLA-DQ, DR (class II) Abs is indicated as I and II, respectively. I/II indicates partial inhibition by both types of mAbs.
e n.d., not done.

FIGURE 2. A, Cytokine profiles. CD4+ T cells could be divided into Th0-like (upper, n = 3) and Th2-like (middle, n = 4) groups. CD8+ TCCs/TCLs show a Tc1-like profile (bottom, n = 4). Left y-axes, IFN-γ level; right y-axes, levels of the other cytokines. B, Chemokine receptor expressions. Representative data for a Th0-like clone (green) and a Th2-like clone (blue) are shown. Black, isotype.
FIGURE 3. Chemotaxis assay. A, Chemotactic activities of normal neutrophils toward IL-8 (upper) and of Th0-like (middle) and Th2-like (bottom) cells toward IP-10 and TARC. Ten randomly selected cells were traced, and the direct distances from the back chamber (y-axis) measured at each time point (X-axis) were plotted. Right, traces. B, Chemotactic activities of Th2-like cells depend on the TARC concentration. C, Chemokine (TARC and IP-10) and chemokine receptor (CCR4 and CXCR3) expressions in the skin lesion.
Results

Phenotypes and TCR Vβs of TCCs and TCLs

Gold-specific TCCs and TCLs were established from PBMCs of a patient with skin rashes at 1 mo after resolution of the eruptions. Among 44 clones and lines, 6 TCCs and 10 TCLs responded well to HAuCl₄ (stimulation index, 2.5–99.0; Table I). All of these clones and lines expressed αβ-TCR and were positive for CD4 and/or CD8. An immunofluorescence study revealed a limited repertoire of TCR Vβs in the TCCs and TCLs, despite the fact that circulating lymphocytes showed diverse usage of 24 TCR Vβs in this patient (data not shown). Specifically, 2 clones/lines had Vβ₁, Vβ₃, Vβ₇, Vβ₁₃.₁, Vβ₁₃.₆, and Vβ₁₇, and 3 clones/lines had Vβ₂, Vβ₅.₁, and Vβ₂₁.₃ (Table I, underlined). Therefore, T cells infiltrating skin rashes shared some Vβs with TCCs and TCLs in vitro. In contrast, we were unable to obtain gold-specific T cells from the other RA patients who had been treated with gold (n = 2) or normal individuals (n = 2) by stimulating their PBMCs with gold.

Functions of gold-specific TCCs/TCLs

The cytokine production profiles of 11 gold-specific TCCs/TCLs were investigated by the CBA assay, as shown in Table I and Fig. 2A. CD4⁺ clones and lines seemed to be classified into two groups, as follows: a high IFN-γ, IL-2, IL-4, IL-5, IL-8, and TNF-α-producing group (Th0-like group; n = 3) and a subset producing low IFN-γ and TNF-α and moderate IL-4 and IL-5, but no IL-2 or IL-8 (Th2-like group; n = 4). All four CD8⁺ TCCs/TCLs produced high IFN-γ, but little or no TNF-α, IL-4, IL-5, or IL-8, apparently showing a Th1-like cytokine profile. A representative CD8⁺ TCC, I-10, exerted 35.5 and 72.5% cytotoxicity toward autologous EBV-infected LCLs in the presence of 10 and 50 μg/ml gold, respectively. These data indicate the presence of various T cell subsets specific to gold among PBMCs.

Functional chemokine receptor expression in gold-specific T cells

Skin-derived chemokines, including TARC, macrophage-derived chemokine, IP-10, and cutaneous T cell attracting chemokine, navigate T cells expressing the corresponding chemokine receptors into the skin from the circulation (13–15). Irrespective of the cytokine profiles, all gold-specific CD4⁺ TCCs/TCLs expressed high levels of CXCR3 and CCR4 and moderate levels of CCR5, CCR9, and CXCR6, but not CCR1, CCR2, CCR3, CCR6, CCR7, CCR8, CXCR1, CXCR2, CXCR4, or CXCR5 (Fig. 2B). To evaluate whether the expressed chemokine receptors were functional, we investigated the chemotactic activities of TCCs/TCLs in response to IP-10 and TARC using a real-time chemotaxis assay system. First, we confirmed linear tracking of freshly prepared neutrophils from a normal individual in response to IL-8 with this system (Fig. 3A, upper). A representative Th0-like TCC, E11F5, migrated, albeit in a zigzag manner, toward IP-10 with the highest chemotactic activity at 10⁻⁸ M. In fact, 10 randomly selected cells reached the back chamber within 30 min (Fig. 3A, middle; supplemental video 1). No activity was detected toward TARC at doses ranging from 10⁻⁷ to 10⁻⁹ M (Fig. 3A, middle; supplemental video 2), because none of the cells ever reached the back chamber. In contrast, a representative Th2-like TCC, F10F5, migrated in response to TARC and showed the highest activity at a dose of 10⁻⁷ M (Fig. 3A, lower, and B) when most of the cells approached the back chamber within 90 min, but did not respond to IP-10 at doses between 10⁻⁷ and 10⁻⁹ M (Fig. 3A, lower). In the skin lesion, epidermis strongly expressed TARC, corresponding to the epidermal accumulation of CCR4⁺ cells (Fig. 3C). In contrast, monocytes around vessels and endothelial cells were highly positive for IP-10, in accordance with perivascular infiltration of CXCR3⁺ cells (Fig. 3C). These results indicate a close relationship between functional chemokine receptor expression and the cytokine profiles of gold-specific TCCs/TCLs.

MHC dependency and MHC restriction in TCCs/TCLs

We investigated MHC restriction in the responses of five representative TCCs (I-1, I-16, F11E5, E10E9, and E10C8) and five representative TCLs (I-8, I-26, I-43, F11D3, and F10F5) to gold in the presence of autologous APCs. The proliferative responses of two CD4⁺ TCCs (F11E5 and E10E9) and two CD4⁺ TCLs (I-8 and I-43) were totally inhibited by a mAb against MHC class II (more than 90%), but not by an anti-HLA-A,B,C mAb (Fig. 4A). In contrast, the proliferation of two CD8⁺ TCCs (I-1 and I-16) and a CD8⁺ TCL (I-26) was completely blocked by the anti-HLA-A,B,C mAb, but not the anti-HLA DR, DQ mAb in the presence of syngeneic APCs (Fig. 4B). F11E5 exhibited a remarkable response to gold in the presence of APC (12,591 ± 1,008) with lower response in the presence of APC fixed with glutaraldehyde (2,514 ± 90) and the culture without gold (2,254 ± 74). In contrast, proliferative responses of F10F5 to gold were comparable between cultures with unfixed APC (5,233 ± 52) and fixed APC (5,610 ± 513; the medium alone, 2,611 ± 168), suggesting a processing-independent pathway. We found unexpected MHC restriction in some TCCs and TCLs. Both anti-HLA-A,B,C and HLA-DR, DQ mAbs substantially, but not completely, inhibited the proliferation of F11D3 (CD8⁺ TCC) and E10C8 (CD4⁺ TCC) in response to gold (Fig. 4C). Proliferation of a CD4⁺ TCC (F10F5) was completely inhibited by the anti-HLA-A,B,C mAb, indicating that the response was restricted by MHC class I (Fig. 4D). These MHC-associated, but unexpected, ways of inhibition suggest that some gold-specific T cells recognize antigenic determinants in a different manner from classical MHC restriction, in which gold is recognized in the groove of MHC.

4 The online version of this article contains supplemental material.
MHC class I allele dependency of gold-specific CD8\(^+\) T cells

Understanding the Ag recognition mechanism of CD8\(^+\) cells in response to drugs is of particular clinical importance due to their special contribution to severe forms of drug allergy (11, 16). Therefore, we further examined the allele dependency of two CD8\(^+\) TCCs (I-1 and I-16) and one TCL (I-26). LCLs were raised as APCs from healthy individuals possessing different MHC haplotypes (donor 1, A2/24, B13/52, Cw3; donor 2, A2/11, B13/60, Cw3/7; donor 3, A2/26, B35/60, Cw3). All of the TCCs/TCLs responded well to syngeneic APCs. I-1 and I-26 responded to allogeneic APCs derived from both donors 2 and 3, whose A or C locus was identical with that of the patient, and APCs from the C locus-matched donor 3, respectively. However, these TCCs and TCLs did not respond to allogeneic APCs with haplotypes that differed from the HLA types of the patient (Fig. 5, A and B). I-16 showed significant proliferation in response to gold, even in the presence of allogeneic APCs without matched haplotypes (Fig. 5C). To confirm whether this proliferative response of I-16 was closely associated with MHC class I molecules expressed on APCs, we examined the inhibition of the response by blocking with an anti-MHC class I mAb. As shown in Fig. 5D, the anti-MHC class I mAb completely inhibited the gold-specific proliferation of I-16 in the presence of syngeneic LCLs as APCs. However, the inhibitory effect on proliferation was marginal with allogeneic APCs. These results imply heterogeneity of the gold-associated Ag recognition in gold-specific T cells, in which some CD8\(^+\) T cells recognize gold independently of MHC class I molecules.

Discussion

In the present study, we established CD4\(^+\) and CD8\(^+\) TCCs and TCLs that were specific for gold. Although CD4\(^+\) and CD8\(^+\) T cells infiltrate skin rashes induced by gold (17), all of the previously reported gold-specific T cells have been restricted to MHC class II (2). However, the generation of functional CD8\(^+\) gold-specific T cells in the present study demonstrates the involvement of these cells in the pathogenesis of gold allergy. We observed that 3 of 15 TCCs and TCLs used V\(\beta\)2, V\(\beta\)5.1, and V\(\beta\)21.3, irrespective of their CD4/CD8 phenotypes, indicating a tendency to use a limited repertoire of TCR V\(\beta\)s. Together with similar observations by ourselves (11) and others (18, 19) for drug allergies, these data suggest preferential affinities of drugs/metals for T cells bearing certain TCR V\(\beta\)s. Despite the limited, but redundant usage of TCR V\(\beta\)s, gold-specific T cells exhibited extensive diversities in their phenotypes, cytokine production profiles, and chemotactic activities. These cells were classified by their cytokine profiles into three groups, namely autoreactive Tc1-like, type 2 cytokine-producing Th2-like, and types 1 and 2 cytokine-producing Th0-like groups. These observations clearly indicate a wide range of pathogeneses in drug/metal allergies.

We found unexpected profiles of inhibition of proliferation by anti-MHC mAbs in some TCCs/TCLs. Among 10 TCCs/TCLs investigated, one CD4\(^+\) TCC and one CD8\(^+\) TCL showed incomplete inhibition of proliferation by both anti-MHC class I and II mAbs. CD4\(^+\) cell type, E10C8, significantly proliferated in response to gold (30,870 ± 2,719; medium alone, 5,150 ± 687). The simultaneous addition of both anti-MHC class I and class II mAbs inhibited partially the proliferation of E10C8 (15,110 ± 2,411) at the levels comparable to the proliferation in the culture with either anti-MHC class I Ab (16,618 ± 1,790) or anti-MHC class II Ab (19,231 ± 1,329). These observations suggest that both MHC class I and II molecules were engaged in the T cell-APC interaction, in an ambiguous manner. Furthermore, in another CD4\(^+\) TCC, the recognition of gold by TCR was paradoxically inhibited by an anti-MHC class I mAb, but not a class II mAb. We also found a nonallele-restricted response of one CD8\(^+\) TCC, because the clone responded to gold in the presence of HLA-unmatched APCs and this response could not be abrogated by an anti-MHC class I mAb. These results indicate that the grove of MHC in APCs, where Ags should ordinarily be settled, would not serve as a conjugating site for gold in these T cells. Ag presentation to F10F5-1 by fixed APC provides evidence of a direct and processing-independent binding of gold to MHC. There are several previous observations that drug-specific T cells are not always restricted by MHC (7, 20–22). One possibility is that the characteristic interactions between T cells and gold-bearing APCs resemble superantigenic reactions to some extent, in which Ags bind to the outside of specific TCR V\(\beta\)s and MHC. Recent findings provide that nickel serves as a direct and peptide-independent linker between certain sites within the TCR \(\alpha\)-chain and the DR \(\beta\)-chain of MHC, resulting in superantigen-like reaction (9, 23, 24), and the similar mechanism may be involved in gold-specific T cell reaction. Alternatively, monomorphic molecules, such as CD39, on the target cell may present haptenized molecules to T cells, raising the possible involvement of molecules other than MHC in the Ag presentation (25). Such a mechanism has been argued for arsonate- and trinitrophenyl-haptenated molecules (26). Focusing on the differences in Ag recognition between drugs/metals and conventional Ags, Pichler et al. (6) recently proposed a novel concept for the mechanism of drug allergy. In this pharmacological-interaction concept, some drugs directly and reversibly bind to TCRs and MHC as pharmacological receptors to embark on signal transduction that results in T cell activation. The pharmacological-interaction concept also explains the limited usage of TCR V\(\beta\)s and unexpected MHC dependency in the gold-specific T cells in the present study.
Our observations provide possible mechanisms for gold allergy, in which not only conventional MHC-restricted, but also promiscuous MHC-unrestricted interactions occur between CD4+ and CD8+ T cells and gold. As a result, various T cell types, including Th0-, Th2-, and Tc1-like T cells, are simultaneously activated. Such an activation mode is completely different from conventional Ag responses, and can explain the variety of clinical manifestations associated with gold allergy. Furthermore, the biased usage of common Vβs between some TCCs and TCLs, and T cells infiltrating skin lesions indicate the pathogenic importance of certain Vβs in the recognition of gold.

Disclosures
The authors have no financial conflict of interest.

References