Mycobactericidal Activity of Human Natural, Monoclonal, and Recombinant Yeast Killer Toxin–like Antibodies

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Human natural (KTAb), murine monoclonal (KTMAb), and single-chain recombinant (KTScFv) candidacidal antibodies represent the internal image of a killer toxin from the yeast *Pichia anomala* (KT), characterized by a wide spectrum of antibiotic activity, exerted a lethal effect against a KT-sensitive multidrug-resistant isolate of *Mycobacterium tuberculosis*. KTMAb and KTScFv were produced by the hybridoma and DNA technologies, respectively, from the spleen lymphocytes of animals immunized with the idiotype of a KT-neutralizing MAb (MAb KT4), while KTAb were purified against MAb KT4 from the vaginal fluid of women infected with *Candida albicans* cells bearing an idiotype-like KT cell wall receptor. Mycobactericidal activity was related to the binding of KTAb, KTMAb, and KTScFv to the cell surface of KT-sensitive bacterial cells and was prevented by specific absorption of KT-like antibodies onto MAb KT4. These data identify a novel potentially useful immunotherapeutic approach to tuberculosis.

Tuberculosis has recently become a reemergent, serious health problem for industrialized countries, as it continues to be for developing countries [1]. For the former, a special threat is the occurrence of multidrug-resistant (MDR) *Mycobacterium tuberculosis* isolates [2]. While requiring the implementation of all known measures to combat the pathogen and the disease, this reemergence also calls for new approaches aimed at an understanding of pathogenesis and protective immunity against the microbial challenger as well as devising novel immunotherapeutic tools and strategies.

In antimycobacterial defense, cell-mediated immunity has always been considered of utmost importance, while little, if any, role has generally been assigned to antibodies. To our knowledge, no anti-mycobacterial antibody endowed with bactericidal activity has ever been reported.

In previous studies, we have shown that serum or secretory immunoprotective yeast killer toxin–like anti-idiotypic antibodies with candidacidal properties (KTIdAb) could be elicited in mice and rats by parenteral or mucosal idiotypic vaccination with a monoclonal antibody (MAb KT4) capable of neutralizing the activity of a wide-spectrum killer toxin (KT) from the yeast *Pichia anomala* against KT-sensitive *Candida albicans* cells [3–7]. While KT was inactivated at physiologic temperature, KTIdAb were shown to mimic KT activity also at 37°C by reacting with a putative idiotype-like KT receptor (KTR), which has been shown to be present even in taxonomically unrelated microorganisms such as *Pneumocystis carinii* [8, 9]. Interestingly, candidacidal antibodies, functionally equivalent to KTIdAb, have been purified from the vaginal fluid of rats experimentally infected with KT-bearing *C. albicans* cells as well as from women, particularly those with recurrent vaginal candidiasis. Like KTIdAb, these human natural KT-like anti-receptor antibodies, termed KTAb, have been shown to kill *C. albicans* cells in vitro, to be neutralized by MAb KT4 in their candidacidal activity, and to confer immunoprotection by passive transfer in experimental models of candidiasis [10]. Recently, we have produced monoclonal KTIdAb (KTMAb) and recombinant KTIdAb in the single-chain format (KTScFv) from the spleen lymphocytes of animals immunized with MAb KT4 [11, 12]. KTMAb and KTScFv were, as KTIdAb, able to kill *C. albicans* cells in vitro, to bind to specific KTR on *C. albicans* cells, and, significantly, to exert a strong therapeutic effect in an in vivo experimental model of vaginal candidiasis. The candidacidal activity of KTMAb and KTScFv was neutralized by MAb KT4.

A schematic representation of the reagents and their reciprocal activities is shown in figure 1. Because of the apparent transphyletic nature of KTR and its potential expression in vivo, we are currently investigating the sensitivity of other medically important microorganisms to the activity of KT and KT-like antibodies.

**Materials and Methods**

Reagents. *P. anomala* KT (ATCC 96603), candidacidal rat IgM KTMAb, and recombinant KTScFv were produced as described elsewhere [10–12]. Human natural candidacidal KTAb were purified by affinity chromatography against MAb KT4 from the vaginal fluid of women.
from the Trudeau Mycobacterial Collection (TMC; Trudeau Insti-
tute, Saranac Lake, NY), was used. Preliminary experiments showed that growth inhibition of M. tuberculosis
was incubated at 25°C to 90°C. For each type of antibody molecule, 1 V region is properly purified from ascites fluid. Each mixture was kept over-night at 4°C.

Preparation and treatment of M. tuberculosis isolate. M. tuberculosis 306, a mutant of isolate H37Rv that proved to be resistant to p-aminosalicylic acid, streptomycin, and isoniazid, obtained from the Trudeau Mycobacterial Collection (TMC; Trudeau Institute, Saranac Lake, NY), was used. M. tuberculosis 306 was cultured in liquid Middlebrook 7H9 medium (Difco, Detroit) containing Tween 80. When the culture reached a predetermined optical density (1 MacFarland), it was diluted 1:100 in sterile distilled water. Aliquots (10 μL) of the bacterial suspension were then added to 90 μL of KT, individual KTAb preparations, KTMAb (100 μg), or KTScFv (10 μg). Identical bacterial suspensions added to the same reagents preincubated with MAb KT4 or to 90 μL of heat-inactivated KT, PBS, irrelevant isotype-matched MAb, or irrelevant ScFv served as the controls. The suspensions were incubated at 25°C (KT in heat-inactivated KT) or 37°C (KT-like antibodies and controls) for 24 and 72 h.

Evaluation of mycobacterial growth index. The BACTEC 460 radiometric system (Becton Dickinson, Cockeysville, MD), was preliminarily used for the assessment of the potential activity of KT and KT-like antibodies by the evaluation of the bacterial growth index. After 24 or 72 h of preincubation, 100 μL of the mycobacterial suspensions added to the different reagents, including controls, was used to inoculate vials containing 4 mL of Middlebrook 7H12B liquid medium that had been previously added to 100 μL of enrichment fluid (Becton Dickinson). The vials were then incubated at 37°C with 5% CO₂ and read daily until the maximum growth index (999) was reached in the controls.

Evaluation of mycobactericidal activity. To assess mycobactericidal activity, a colony-forming unit assay was done from the bacterial suspensions added to a selected KTAb (preparation 1), KTMAb (100 μg), KTScFv (10 μg), or the same reagents preincubated with MAb KT4, as well as PBS, irrelevant isotype-matched MAb, or irrelevant ScFv as controls, after 72 h of incubation. Ten microliters of each bacterial suspension was diluted 1:30 in PBS, and then 100 μL was dispensed on the surface of Middlebrook 7H11 agar plates (Difco). The plates were then incubated at 37°C with 5% CO₂ and examined for colony growth. Each experiment was done in triplicate for statistical purposes.

Immunofluorescence studies. The immunofluorescence assays were done by using biotinylated reagents. KT (0.250 mg/mL), purified KTAb (preparation 1; 0.175 mg/mL), KTMAb (1 mg/mL), and KTScFv (0.5 mg/mL) were biotin-labeled by using a previously described procedure [11, 12]. One milliliter of the broth culture of M. tuberculosis TMC 306 was centrifuged (2200 g for 20 min), and the cell pellet was washed twice with sterile PBS. The pellet was then suspended in PBS; next, 20 μL of the bacterial suspension was put into each well of an immuno-fluorescence slide. The pellet was then suspended in PBS; next, 20 μL of the bacterial suspension was put into each well of an immuno-fluorescence slide. After 24 h of contact, then evaluating the growth index compared with KTMAb (100 μg), KTScFv (10 μg), or the same reagents preincubated with KTMAb (100 μg), KTScFv (10 μg), or KTAb (preparation 1) as well as KTMAb had a clear mycobactericidal effect, as shown by the colony-forming unit assay, with a comparable percentage of decrease in colony number with respect to controls (90.50 ± 1.5, KTAb;
Figure 2. Growth of multidrug resistant isolate of *Mycobacterium tuberculosis* TMC 306 after 72 h of treatment of standardized bacterial inoculum with *Pichia anomala* killer toxin (KT) compared with heat-inactivated KT used as control (A); human natural affinity chromatography–purified yeast KT-like antibodies (KTAb) from 2 different persons compared with PBS used as control (B); yeast KT-like monoclonal anti-idiotypic antibody (Mab K10) compared with irrelevant isotype-matched MAb used as control (C); yeast KT-like recombinant anti-idiotypic antibody (KTScFv H6) compared with irrelevant ScFv anti-idiotypic antibody used as control.
Figure 3. Immunofluorescence visualization of putative yeast killer toxin cell wall receptors on *Mycobacterium tuberculosis* TMC 306 cells by biotinylated yeast killer toxin–like monoclonal anti-idiotypic antibody (MAb K10). Reactivity is mostly related to microbial aggregates, although detectable even in single cells.

81.30 ± 1.75, KTMAb; 90.33 ± 0.35, KTScFv). This mycobactericidal activity was completely abrogated (0 decrease in colony-forming units) by preincubation of the same reagents with MAb KT4.

KT, KTAb, KTMAb, and KTScFv preparations were seen to bind to the *M. tuberculosis* TMC 306 cell surface, as shown by immunofluorescence. Figure 3 is an example of mycobacterial cell staining with a biotinylated KTMAb preparation. The immunofluorescence reactivity of each biotinylated reagent was neutralized by previous mixing with MAb KT4. All biotinylated reagents (KT, KTAb, KTMAb, and KTScFv) competed with their own unlabeled form as well as with each other for binding to the surface of mycobacterial cells. When adsorbed with KT-susceptible *C. albicans* cells, each reagent lost its ability to react with *M. tuberculosis* in the same experimental conditions.

Discussion

Here we report that yeast killer toxin–like natural (human), or artificial (murine monoclonal and recombinant) antibodies, previously characterized for being candidacidal [10–12], do also exert a bactericidal activity in vitro against an MDR isolate of *M. tuberculosis*. Data not shown here also demonstrated that the above reagents are able to kill non-MDR isolates of *M. tuberculosis*.

KT-like antibodies also exerted an antimycobacterial effect after 24 h of contact with MDR *M. tuberculosis* TMC 306 cells, even though the inhibitory activity was more significant after 72 h of exposure. Significantly, KTAb preparations, including the ones from 3 other patients previously characterized [10], showed a growth index decrease (calculated on day 13, when the untreated control reached the maximum growth) that ranged from ~60% to >90% depending on the individual KTAb preparation (data not shown). Differences in potency were likely due to differing amounts of bactericidal KTAb in each preparation, which has been purified by affinity chromatography and has been shown to contain various anti-idiotypic antibodies with different isotypes and proportion of KT-mimicking, true internal image antibodies [10]. It must be noted that the most active anti-*M. tuberculosis* KTAb preparations used throughout this study (preparations 1 and 2) were also the most active against *C. albicans* [10], indirectly supporting a common receptor and mechanism of action. Nonetheless, more homogeneous and reproducible results were obtained when standardized reagents, such as KTMAb and KTScFv, were used under the same experimental conditions. The reduction in the number of colony-forming units after treatment of the mycobacterial suspensions with the above reagents in comparison with those enumerated before the treatment demon-
strates the mycobactericidal activity of KT-like antibodies (data not shown).

The neutralization of the mycobactericidal effect of KT, KTAb, KTMAb, and KTScFv by preincubation with anti-KT MAb KT4 and the similar pattern of fluorescence observed on the mycobacterial cell wall after treatment with each biotinylated reagent suggest that KT and KT-like antibodies bind to a specific KTR present on the mycobacterial cell wall. Immunofluorescence competition experiments also indicate that each KT-like antibody bound to the same KTR as does KT. Importantly, this KTR should be homologous to that previously identified on C. albicans cell wall [13], as demonstrated by the immunofluorescence experiments done on M. tuberculosis cells with each reagent preadsorbed with KT-susceptible C. albicans cells. Since a putative KTR has also been shown to occur on the surface of KT-susceptible P. carinii organisms, a possibility exists that KTAb, KTMAb, and KTScFv display their antimicrobial activity also against this organism recently described as a fungus [9]. Finally, our data show that the pattern of multiresistance of M. tuberculosis is not related to its susceptibility to KT and KT-mimicking KTR ligands, KTAb, KTMAb, and KTScFv.

The presence of transphyletic, cross-reactive antigens is not uncommon among microorganisms. The main feature of KTR seems to reside in its capacity to induce antibodies that, by their KT mimicry, possess microbicidal properties. Immunofluorescence studies suggest that the putative transphyletic KTR is expressed on the M. tuberculosis cell surface and is readily accessible to antibodies. Considering the demonstrated antigenicity of KTR [10], this would theoretically imply that mycobactericidal antibodies could be produced during M. tuberculosis infection, a hypothesis that warrants further investigation [14].

Human recombinant KTAb, KTMAb, or KTScFv might be engineered to obtain synthetic derivatives with mycobactericidal properties, which, by appropriate delivery systems, could represent a new, potentially powerful tool, particularly in the fight against MDR M. tuberculosis infections.

References