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ORIGINAL PAPER

From *Pichia anomala* killer toxin through killer antibodies to killer peptides for a comprehensive anti-infective strategy

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Abstract "Antibiobodies", antibodies (Abs) with antibiotic activity, internal image of a *Pichia anomala* killer toxin (*Pa*KT) characterized by microbicidal activity against microorganisms expressing β-glucans cell-wall receptors (*Pa*KTRs), were produced by idiotypic vaccination with a *Pa*KT-neutralizing monoclonal Ab (*Pa*KT-like Abs) or induced by a protein-conjugated β-glucan. Human natural *Pa*KT-like Abs (*Pa*KTAbs) were found in the vaginal fluid of women infected with KT-sensitive microorganisms. Monoclonal and recombinant *Pa*KT-like Abs, and *Pa*KTAbs proved to be protective against experimental candidiasis, cryptococcosis and aspergillosis. A killer decapeptide (KP), synthesized from the sequence of a recombinant *Pa*KT-like Ab or produced in transgenic plants, showed a microbicidal activity in vitro, neutralized by β-glucans, a therapeutic effect in vivo, against experimental mucosal and systemic mycoses, and a prophylactic role *in planta*, against phytopathogenic microorganisms, respectively. KP showed fungicidal properties against all the defective mutants of a *Saccharomyces cerevisiae* library, inclusive of strains recognized to be resistant to conventional antifungal drugs. KP inhibited in vitro, ex vivo and/or in vivo HIV-1 and Influenza A virus replication, owing to down-regulation of CCR5 co-receptors, physical block of the gp120-receptor interaction and reduction in the synthesis of glycoproteins, HA and M1 in particular. KP modulated the expression of costimulatory and MHC molecules on murine dendritic cells, improving their capacity to induce lymphocyte proliferation. KP, proven to be devoid of cytotoxicity on human cells, showed self-assembly-releasing hydrogel-like properties, catalyzed by β 1,3 glucan. *Pa*KT's biotechnological derivatives may represent the prototypes of novel antifungal vaccines and anti-infective drugs characterized by different mechanisms of action.

Keywords *Pichia anomala* · Yeast killer toxin · Microbicidal antibodies · Anti-infective peptides · Antifungal vaccines

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The yeast killer phenomenon may be defined as the lethal activity exerted by killer toxins (KTs) secreted by self-immune killer yeasts on sensitive yeasts presenting specific cell wall receptors (KTRs) (Bevan and Makower 1963). Yeast killer systems are characterized by different genetic determinants, horizontally or vertically acquired, i.e., *Saccharomyces cerevisiae* and *Ustilago maydis* rely on double stranded RNA genomes encapsidated in intracellular non infectious virus-like particles, *Kluyveromyces lactis* on pair of linear double stranded DNA

plasmids, *Pichia anomala*, lately proposed to be redefined as *Wickerhamomyces anomalus*, and *Williopsis mrakii* on chromosomal genes. KTs may display different mechanisms of action in various yeast systems, i. e. increase of membrane permeability to ions (*S. cerevisiae* and *P. kluyveri*), induction of endonucleolytic cleavage of single stranded RNA and DNA and double stranded DNA (*U. maydis*), inhibition of adenylate cyclase (*K. lactis*), interaction with β-glucan KTRs on sensitive cells (*P. anomala* and *W. mrakii*) (Magliani et al. 1997a).

A selected strain of *P. anomala* (ATCC 96603) has proven to display a wide spectrum of microbicidal activity against yeasts, molds, aerobic and anaerobic bacteria, aerobic actinomycetes, lipophilic yeasts, and achlorophyllous microorganisms. This was the first demonstration that a killer yeast may display a lethal activity against taxonomically unrelated eukaryotic and prokaryotic pathogenic microorganisms (Polonelli and Morace 1986).

A KT of *P. anomala* ATCC 96603 (*Pa*KT) proved to exert a therapeutic activity by topical application against superficial experimental infections caused by *Malassezia furfur* in guinea pigs (Polonelli et al. 1986). Therapy by parenteral administration was unfeasible because *Pa*KT proved to be antigenic, toxic and very labile at physiological pH and temperature values (Pettoello-Mantovani et al. 1995).

The concept of duality, originated in ancient Chinese philosophy and metaphysics, describes two primal opposing but complementary forces found in all things in the universe, and postulates that in nature no entity is so pure that it does not contain complementary opposites in a diminished form. Thus, any dichotomy can be seen as its opposite when viewed from another perspective and the categorization is seen as one of convenience. Looking at the functional topography of an antibody (Ab) molecule, many different antigenic determinants or epitopes (E) may be found in the constant region (isotopes and allotopes) and, particularly, in the variable region (idiotopes). The ensemble of the idiotopes constitutes the private idiotype (Id) or paratope (P) (antigen binding site) of each Ab. In this perspective, the idiotypic network theory formulated by Jerne (1974) was based on the dual character of Abs which recognize the specific antigen through the paratope and are immunogenic themselves by virtue of their allotopes and idiotopes.

According to this view, we have exhausted the idiotypic network of the *P. anomala* killer phenomenon. The interaction between the functional E of the wide-spectrum microbicidal *Pa*KT and specific β1,3 D-glucan cell-wall receptors (*Pa*KTR) in sensitive yeasts should be mirrored by the binding between the Id of a *Pa*KT neutralizing monoclonal Ab (mAb KT4) and its anti-idiotype (anti-Id) postulating that, while Id may mimic *Pa*KTR acting as a vaccine, anti-Ids may mimic *Pa*KT acting as killer Abs characterized by antibiotic activity (antibiobodies) (Polonelli and Morace 1987; Polonelli et al. 1991, 1993).

When used as immunological probes, polyclonal *Pa*KT-like Abs, elicited in mice and rabbits by immunization with mAb KT4, showed differential immunofluorescence reactivity with *Candida albicans* and mammalian cells. Specific *Pa*KTRs were occurring exclusively on the surface of microbial cells and were preferentially located on germ tubes and budding scars, where β-glucans were exposed (Polonelli et al. 1990). *Pa*KT-like Abs proved to exert fungicidal activity against *C. albicans* cells constituting the first demonstration that an Ab can directly kill a microorganism in absence of complement or effector cells (Polonelli et al. 1991).

A dual anti-infective approach, based on idiotypic mimicry of *Pa*KT and *Pa*KTRs, respectively, could be then envisaged: anti-Id therapy, i.e. *Pa*KT-like Abs directly injected into infected animals, and Id vaccination, i.e. immunization with mAb KT4 for in vivo production of *Pa*KT-like Abs protecting animals against experimental infections (Magliani et al. 1997a). Parenteral immunization with mAb KT4 proved to protect rats against experimental systemic candidiasis in comparison to infected non immunized control animals (Polonelli et al. 1993). Analogously, intravaginal immunization with mAb KT4 was able to protect rats against experimental vaginal candidiasis. Significantly, the vaginal fluids of vaccinated rats transferred passive protection, mediated by secretory *Pa*KT-like Abs, to naive animals against vaginal candidiasis (Polonelli et al. 1994).

A new principle of vaccination was claimed that is conceptually different from previous conventional, recombinant, DNA and even anti-Id vaccination, devoted to elicit protective Abs directed to the native, recombinant, encoded or mimicked virulence factor of the etiologic agent. In idiotypic vaccination, the immunogen is constituted by an Id representing the

internal image of a receptor specific for a foreign molecule killing the etiologic agent and inducing antibiobodies (Magliani et al. 1997a).

Significantly, intravaginal and intragastric administration of *Pa*KT-sensitive yeasts in rats intravaginally immunized with mAb KT4, and challenged with *C. albicans* cells, produced a booster effect in the production of *Pa*KT-like Abs. Thus, the immune system recognized the Id of mAb KT4 as *Pa*KTR (Polonelli et al. 1996). Coherently, immunization with *C. albicans* cells treated with DTT and K proteinase prolonged survival and prevented kidney invasion in mice in comparison with immunization with untreated yeast cells. Only *C. albicans* cells devoid of superficial mannoproteins and exposing β1,3 D-glucan (*Pa*KTR) were protective (Bromuro et al. 2002).

This assumption led to the production of a β1,3 D-glucan based "universal" antifungal vaccine. Laminarin (soluble β1,3 D-glucan) of algal origin (Lam) was selected to attest the transphyletic potential of a vaccine based on Abs directed to a viability-critical and immutable cell wall component shared by all fungal pathogens. Vaccination with Lam conjugated to a diphtheria toxoid (CRM) induced a strong Ab-mediated anti-*Candida* protection in a murine experimental model of disseminated infection. Specific anti β-glucan Abs conferred passive protection to naive mice against disseminated candidiasis. Abs raised by intravaginal vaccination with Lam-CRM conjugate significantly accelerated the resolution of a rat vaginal infection with *C. albicans.* Anti β-glucan Abs were involved in immunoprotection and conferred passive protection to naive mice against vaginal candidiasis comparable to the therapeutic effect exerted, in the same experimental conditions, by fluconazole. In vitro growth of *C. albicans* and *Aspergillus fumigatus* was significantly restricted by the anti-β1,3 D-glucan Abs, and Lam-CRM vaccination significantly prolonged the survival of mice subjected to a systemic challenge with *A. fumigatus* (Torosantucci et al. 2005).

A mAb specific to β1,3 D-glucan inhibited the growth and the capsule formation in different strains of *Cryptococcus neoformans* (Rachini et al. 2007).

An intriguing corollary in the idiotypy of the *P. anomala* killer phenomenon was the existence of human natural *Pa*KT-like Abs (*Pa*KTAbs). *Pa*KTAbs specific to *Pa*KTR should be elicited, among others, in individuals infected by *Pa*KT-sensitive microorganisms. Women vaginally infected or colonized by *Candida* spp. showed indeed the presence of *Pa*KTAbs in their vaginal fluid. Human vaginal *Pa*KTAbs protected by passive transfer rats against experimental vaginal candidiasis. This was the first demonstration that human natural anti-KTR Abs may play a role in anti-infective immune defense (Polonelli et al. 1996).

*Pa*KTAbs inhibited in vitro the growth of a multidrug resistant strain of *Mycobacterium tuberculosis*, thus exceeding the spectrum of activity of conventional antibacterial drugs, and *Pneumocystis carinii* infectivity to nude rats. This activity was significatively inhibited by mAb KT4 (Conti et al. 1998; Seguy et al. 1997b).

In order to have unlimited availability and absolute reproducibility of reagents, *Pa*KT-like Abs were produced in the monoclonal (*Pa*KTmAb), phage displayed (*Pa*KTph) and soluble single chain fragment variable (*Pa*KTscFv) recombinant formats. Likewise *Pa*KT, *Pa*KTAbs in the different formats displayed wide spectrum microbicidal activity although *Pa*KTph and *Pa*KTscFv were devoid of the constant region. *Pa*KTmAb and *Pa*KTscFv protected rats against experimental vaginal candidiasis and the therapeutic effect was superior to the one exerted, in the same experimental conditions, by fluconazole (Magliani et al. 1997b). *Pa*KTmAb inhibited in vitro *A. fumigatus* conidial germination and was therapeutic in a murine model of invasive aspergillosis (Cenci et al. 2002). Aerosol of *Pa*KTmAb protected rats against experimental pneumocystosis. The therapeutic activity was similar to the one exerted, in the same experimental conditions, by the conventional anti-*Pneumocystis* drug pentamidine (Seguy et al. 1997a).

Adherence of *Streptococcus mutans* to dental surfaces is prejudicial for pathogenesis of dental caries caused by plaque formation (Loesche 1986). *Pa*KTmAb and *Pa*KTscFv proved to display a strong activity against *S. mutans* in vitro and in an ex vivo model realized to investigate the potential of drugs to inhibit the formation or the reproduction of dental plaque in controlled conditions (Conti et al. 2002).

*Pa*KTscFv were expressed in *Streptococcus gordonii*, a generally recognized as safe bacterium colonizing mucosal surfaces. Transformants of *S. gordonii* expressing *Pa*KTscFv on the surface displayed activity in vitro against *C. albicans* cells. Increasing recombinant bacteria/yeasts ratios resulted

in increased yeast killing activity. Supernatants of *S. gordonii* secreting recombinant *Pa*KTscFv displayed in vitro candidacidal activity in comparison to the supernatants of parental streptococci. This candidacidal activity was significantly inhibited by mAb KT4. Both colonizing *S. gordonii* transformants, superficially expressing or secreting candidacidal *Pa*KTscFv, protected rats against experimental vaginal candidiasis. The therapeutic activity exerted by transformants secreting *Pa*KTscFv was superior to that exerted, in the same experimental conditions, by fluconazole (Beninati et al. 2000).

In order to obtain the smallest peptide still maintaining the biological activities of *Pa*KT and *Pa*KT-like Abs, *Pa*KTscFv was sequenced, and a series of two residues displaced decapeptides reproducing its entire sequence was synthesized. A decapeptide (P6, EKVTMTCSAS) was selected according to highest candidacidal activity observed by Colony Forming Unit (CFU) assays. As alanine scanning may display the functional role of each residue within a peptide, the substitution E-A (AKVTMTCSAS) showed an enhancement of the activity of the engineered killer peptide (KP). A scramble decapeptide (SP, MSTAVSKCET) constituted by the same residues with different sequence was proven to be devoid of any candidacidal activity (Polonelli et al. 2003).

The in vitro activity of KP against *C. albicans* cells was time and dose dependent, and differently neutralized by β-glucans (Magliani et al. 2004a). The candidacidal effect was rapid and preferentially abolished by β1–3 glucan (laminarin), the main component of *Pa*KTR, rather than β1–6 glucan (pustulan). Intravaginal administration of KP eradicated vaginal candidiasis in rats. The therapeutic effect was similar to the one exerted, in the same experimental conditions, by fluconazole, even though KP proved to be active also on fluconazole-resistant strains of *C. albicans* (Polonelli et al. 2003). Thus, KP's therapeutic effects were exerted beyond the activity of conventional antifungal drugs. Parenteral administration of KP protected against systemic candidiasis also SCID mice, attesting that its antibiotic activity was sufficient for therapeutic purposes (Polonelli et al. 2003).

KP exerted sterilizing activity on sanded acrylic resin discs previously contaminated with *C. albicans* cells (Manfredi et al. 2007). This finding may be of significance in oral microbiology presuming KP potential against candidal biofilms.

KP treatment produced morphological effects on *C. albicans* cells. Cell wall swollen, middle electron-dense region and plasma membrane collapse were observed, while nucleus fragmentation was reminiscent of treatment with apoptotic agents, such as acetic acid. Preliminary studies, carried out with *C. albicans* mutants and DNA microarrays, have shown that the response to oxidative stress and ion transport within the yeast cell may be involved in the mechanism of action of KP (Magliani et al. 2008).

KP killed in vitro *C. neoformans* cells, and impaired the production of specific yeast virulence factors. Interference with protease activity and, particularly, capsule formation could be of relevance for therapeutic purposes. Significantly, KP treatment reduced fungal burden in normal and immunosuppressed mice experimentally infected with *C. neoformans* cells, and was effective in protecting immunosuppressed animals from an otherwise lethal infection (Cenci et al. 2004).

KP was fungicidal in vitro against the dimorphic fungus *Paracoccidioides brasiliensis.* Killer activity was also displayed by a peptide constituted by the same ten residues in the D-isomeric form, a composition which should make the decapeptide resistant to proteases. In an animal model of systemic paracoccidioidomycosis, KP exerted therapeutic activity sterilizing the tissues, and preserving the anatomical structure of the target organs (lungs, kidneys, liver) (Travassos et al. 2004).

KP displayed activity in vitro against *Leishmania infantum* and *L. major.* The activity was leishmanicidal, dose and time dependent and caused gross morphology alterations in the treated cells, as visualized by transmission electron microscopy (Savoia et al. 2006).

KP inhibited the growth of *Acanthamoeba castellanii.* The activity against this important agent of keratitis was exerted in vitro either at 25° or 37°C as well as onto the surface of contaminated contact lens (Fiori et al. 2006).

Chimeric virus particles displaying the killer peptide exerted in vitro and *in planta* inhibitory activity against bacterial and fungal pathogens. *Nicotiana benthamiana* plants were inoculated with the viral constructs pPVX-KP, bearing KP, and pPVX-SP, bearing SP, as a control. Other than representing potential biofarms, transgenic plants expressing the killer peptide were resistant to infections caused by

phytopathogenic agents. Chlorotic and necrotic symptoms were observed in the upper leaves after the infection with *Pseudomonas syringae* pv. *tabaci* only in plants secreting SP (Donini et al. 2005).

KP proved to modulate phenotype and function of murine dendritic cells. Binding occurred through DC-SIGN, MHC class II, and CD16/32 molecules and modulated the expression of costimulatory and MHC molecules on murine dendritic cells improving their capacity to induce lymphocyte proliferation and drive a Th1 protective response (Cenci et al. 2006).

Surprisingly, KP showed sequence similarity with critical segments in loops of HIV-1 envelope polyprotein precursor from several isolates. This finding stimulated to investigate KP inhibitory potential on HIV endogenous and exogenous infections. KP displayed ex vivo activity on HIV-1 replication in PBMCs cultures obtained from an AIDS patient in acute phase of infection. The antiretroviral activity of KP was nearly ten times greater than the one exerted, in the same experimental conditions, by azidothymidine. KP displayed in vitro activity also on HIV-1 replication in stimulated PBMCs from healthy donors exogenously infected with a lymphocytotropic or a monocytotropic HIV strain. While Bal monocytotropic HIV-1 strain is using CCR5, IIIb lymphocytotropic HIV-1 strain exploits CXCR4 chemokine co-receptor to infect the cells. Down-regulation of CCR5 co-receptor, and/or physical block of the gp120-receptor interaction are possible mechanisms of KP activity (Casoli et al. 2006).

KP proved to be effective on influenza A virus replication in infected *Rhesus* monkey kidney cells. The intracellular antiviral activity was approximately exerted at the 4th hour of infection. Treatment with KP affected the synthesis of Influenza A late viral proteins, particularly hemagglutinin and M1. In vitro inhibitory activity was also exerted on the replication of a H7N3 avian virus and a clinical isolate (H3N2) of Influenza A virus characterized by amantadine resistance. Importantly, KP exerted a therapeutic effect, as demonstrated by prolonged survival time and reduced viral load in the lungs, in mice infected with a neurovirulent strain of influenza A virus (Conti et al. 2008a).

KP displayed activity in vitro against all deleted mutants of a *S. cerevisiae* wide-genome library. None of the ~4,800 mutants deleted for nonessential genes, inclusive of strains resistant to conventional antifungal drugs, exhibited decreased susceptibility to the synthetic killer peptide. The results may reflect the peculiar mechanism of action of KP and claim the possible avoidance of vital resistant mutants (Conti et al. 2008b).

Noteworthy, KP was observed to display intrinsic self-assembling properties, catalyzed by laminarin. Accordingly, KP was considered to be the first hydrogel-like therapeutant. KP proved to aggregate also on the surface of *C. albicans* cells, particularly on budding scars where β1–3-glucans are exposed. While the self-assembled state may provide protection against proteases and the slow kinetic of dissociation assures a release of the active form over time, the receptor affinity may be responsible for targeted delivery (Pertinhez et al. 2009).

In conclusion the idiotypic mimicry of evolutionarily selected competing factors among microorganisms, such as *Pa*KT, led to the discovery of functionally homologous natural microbicidal Abs, characterized by similar wide-spectrum peculiar fungicidal effect mediated by β-glucan interaction and interference with fungal virulence factors. *Pa*KT-like recombinant Abs have resulted propaedeutic to the synthesis of KP that has proven to display in vitro, ex vivo and/or in vivo antifungal, antibacterial, antiparasitic, antiviral, and immunomodulatory activities mediated by different mechanisms of action. KP, likewise SP, showed no toxicity even at high concentrations in all tested mammalian cells growing in culture including lymphocytes (Magliani et al. 2004b).

The easiness of production and low cost of small sized synthetic peptides, the possibility of peptide engineering and chemical optimization associated to new delivery mechanisms are expected to give rise to a new generation of comprehensive anti-infective agents.

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