Antibody Complementarity-Determining Regions (CDRs) Can Display Differential Antimicrobial, Antiviral and Antitumor Activities

Luciano Polonelli1, José Pontón2, Natalia Elguezabal2, María Dolores Moragues3, Claudio Casoli4, Elisabetta Pilotti5, Paola Ronzi2, Andrey S. Dobroff6, Elaine G. Rodrigues6, Maria A. Juliano7, Domenico Leonardo Maffei1, Walter Magliani1, Stefania Conti1, Luiz R. Travassos6*

1 Dipartimento di Patologia e Medicina di Laboratorio, Sezione di Microbiologia, Università degli Studi di Parma, Parma, Italy, 2 Dipartimento di Immunologia, Microbiologia y Parasitología, Facultad de Medicina y Odontología, Universidad del País Vasco, Bilbao, Vizcaya, Spain, 3 Departamento de Enfermería I, Universidad del País Vasco, Bilbao, Vizcaya, Spain, 4 Dipartimento di Scienze Cliniche L. Sacco, Sezione di Malattie Infettive e di Immunopatologia, Università di Milano, Milano, Italy, 5 Dipartimento di Clinica Medica, Nefrologia e Scienze della Prevenzione, Università degli Studi di Parma, Parma, Italy, 6 Unità di Oncologia Experimental, Dipartimento di Microbiologia, Immunologia e Parasitologia, Universidade Federal de São Paulo, São Paulo, Brazil, 7 Departamento de Biofísica, Universidade Federal de São Paulo, São Paulo, Brazil

Abstract

Background: Complementarity-determining regions (CDRs) are immunoglobulin (Ig) hypervariable domains that determine specific antibody (Ab) binding. We have shown that synthetic CDR-related peptides and many decapeptides spanning the variable region of a recombinant yeast killer toxin-like antiidiotype Ab are candidacidal in vitro. An alanine-substituted decapetide from the variable region of this Ab displayed increased cytotoxicity in vitro and/or therapeutic effects in vivo against various bacteria, fungi, protozoa and viruses. The possibility that isolated CDRs, represented by short synthetic peptides, may display antimicrobial, antiviral and antitumor activities irrespective of Ab specificity for a given antigen is addressed here.

Methodology/Principal Findings: CDR-based synthetic peptides of murine and human monoclonal Abs directed to: a) a protein epitope of Candida albicans cell wall stress mannanprotein; b) a synthetic peptide containing well-characterized B-cell and T-cell epitopes; c) a carbohydrate blood group A substance, showed differential inhibitory activities in vitro, ex vivo and/or in vivo against C. albicans, HIV-1 and B16F10-Nex2 melanoma cells, conceivably involving different mechanisms of action. Antitumor activities involved peptide-induced caspase-dependent apoptosis. Engineered peptides, obtained by alanine substitution of Ig CDR sequences, and used as surrogates of natural point mutations, showed further differential increased/unaltered/decreased antimicrobial, antiviral and/or antitumor activities. The inhibitory effects observed were largely independent of the specificity of the native Ab and involved chiefly germline encoded CDR1 and CDR2 of light and heavy chains.

Conclusions/Significance: The high frequency of bioactive peptides based on CDRs suggests that Ig molecules are sources of an unlimited number of sequences potentially active against infectious agents and tumor cells. The easy production and low cost of small sized synthetic peptides representing Ig CDRs and the possibility of peptide engineering and chemical optimization associated to new delivery mechanisms are expected to give rise to a new generation of therapeutic agents.

Citation: Polonelli L, Pontón J, Elguezabal N, Moragues MD, Casoli C, et al. (2008) Antibody Complementarity-Determining Regions (CDRs) Can Display Differential Antimicrobial, Antiviral and Antitumor Activities. PLoS ONE 3(6): e2371. doi:10.1371/journal.pone.0002371

Editor: Hany A. El-Shemy, Cairo University, Egypt

Received February 1, 2008; Accepted April 29, 2008; Published June 11, 2008

Copyright: © 2008 Polonelli et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: Department of Education, Universities and Research, Basque Government (grant IT-264-07), FAPESP 06/50634-2 grant, São Paulo, Brazil; Istituto Superiore di Sanità, National Research Project on A.I.D.S. (grants 50G.30 and 40D.14, ELVIS Italian Network on LTNP), and the Cariparma Banking Foundation (grant 2004.0190). Funding agencies had no role in the design and development of the research work. LRT and EGR are recipients of a research fellowship from the Brazilian National Research Council (CNPq).

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: luiztravassos@gmail.com

Introduction

Immunoglobulins are composed of polymorphic heavy and light chains. The idiotypic variability is related to the diversity of the antigen binding site and in particular to the hypervariable domains called complementarity-determining regions (CDRs). There are 6 CDRs in both variable regions of light (V̂ L) and heavy chains (V̂ H) with background variability on each side of the CDRs. Antibodies (Abs) of different specificities can assemble identical V̂ L domains with different V̂ H domains. The framework sequences between CDRs can be similar or identical.

Idiotypic vaccination with a murine monoclonal Ab (mAb KT4), that neutralizes the wide-spectrum antimicrobial activity of a yeast killer toxin (KT) against eukaryotic and prokaryotic microorganisms presenting specific cell wall receptors (KTR), elicited the production of a special sub-set of antiidiotype Abs (KT-antiId) characterized by
in vitro microbicidal and in vivo therapeutic effects [1]. Abs functionally mimetizing KT were detected in the serum or secretions of animals and humans experimentally or naturally infected with KTR-bearing microorganisms (KT-Abs) and have been produced in the monoclonal (KT-mAb) and recombinant (KT-scFv) formats. KT-Abs, KT-mAb and KT-scFv conferred passive immunoprotection in experimental models of mucosal and systemic fungal infections [2–4].

The peptides corresponding to the CDRs of KT-scFv, as well as a series of two-residue displaced overlapping decapeptides spanning the variable region were synthesized. All the synthetic CDRs and most of the related decapeptides showed a fungicidal effect against Candida albicans in vitro [5]. The most in vitro and in vivo active fragment (P6), including seven amino acids of the framework region and the first three residues of the light chain CDR1 of KT-scFv, was well represented among the sequences of many unrelated Abs. A killer decapeptide (KP) generated by alanine substitution of the last residue in H2 (K16A) resulted in activities similar to H2, but some showed modulating effects (data not shown). Most H2 alanine-substituted derivatives (Table 2) had activities similar to H2, but some showed modulating effects (data not shown). Most H2 alanine-substituted derivatives (Table 2) had activities similar to H2, but some showed modulating effects (data not shown).

Anticandidal activities of CDR-based synthetic peptides

The in vitro microbicidal activity of mAb C7 and mAb pc42 and mAb HuA CDR-based synthetic peptides at 100 μg/ml, and the EC₅₀ of CDRs that exhibited a significant activity against C. albicans UP10 are shown in Table 1. The most active CDR peptides were mAb pc42 L1, mAb C7/pc42 H2 and HuA L3 (Fig. 1A). Similar results were obtained with C. albicans NCPF 3153 (data not shown). Most H2 alanine-substituted derivatives (asd) had activities similar to H2, but some showed modulating effects (Table 2). Substitution of the last residue in H2 (K16A) resulted in a fundamental loss of activity whereas substitution of the previous amino acid (F15A) gave rise to the highest candidacidal derivative.

Figure 1. Anticandidal activities of mAb CDRs. A. In vitro candidacidal activity of three different CDR peptides (HuA L3, pc42 L1, C7/pc42 H2) against Candida albicans UP 10 cells as shown by CFU assay. C. albicans cells were treated with 100 μg/ml of each peptide in distilled water (upper) in comparison with distilled water only (lower). Each plate is representative of an assay carried out in triplicate; B. Effect of mAb C7/pc42 H1 and H2 and mAb C7 L1 CDRs on the survival curve of mice (11–12 animals/group) infected intravenously with 5 × 10⁶ yeast cells of C. albicans. The survival curve of mAb C7 L1-treated animals was significantly different (p = 0.01) from that of control mice. MST ± SE mean survival time ± standard errors. doi:10.1371/journal.pone.0002371.g001

A mAb (C7), raised against C. albicans cell wall stress mannanprotein, a major target of human secretory IgA in the course of oral and vaginal candidiasis, has been recently described [11]. As a polyclonal IgM, mAb C7 cross-reacted with cell wall proteins of C. albicans Als3 and enolase, as well as with the nuclear pore complex Nup88 [12,13]. MAb C7 is the first Ab able to exert three different antifungal activities against C. albicans, such as inhibition of germination and adhesion and a direct fungicidal effect which extended to C. lusitaniae, Cryptococcus neoformans, Aspergillus fumigatus, and Scedosporium prolificans, suggesting a pleiotropic mode of action [11]. Recently, mAb C7 proved to be protective in a murine model of systemic candidiasis [14].

As a proof of concept of the extrinsic potential of Ab fragments, we now report on the differential antimicrobial, antiviral and antitumor activities of synthetic peptides with sequences identical to CDRs of the light chain (L1, L2 and L3) and heavy chain (H1, H2 and H3) of: a) mAb C7; b) mouse mAb pc42 (IgM), directed to a synthetic peptide containing the surface antigen of hepatitis B virus and the T-helper-cell epitope from the circumsporozoite protein of Plasmodium falciparum, sharing H1 and H2 with mAb C7 [15]; and c) a human IgM mAb (HuA) specific for difusosyl human blood group A substance sharing no CDR homology with either mAb C7 or mAb pc42 [16].

Results

Anticandidal activities of CDR-based synthetic peptides

The in vitro microbicidal activity of mAb C7, mAb pc42 and mAb HuA CDR-based synthetic peptides at 100 μg/ml, and the EC₅₀ of CDRs that exhibited a significant activity against C. albicans UP10 are shown in Table 1. The most active CDR peptides were mAb pc42 L1, mAb C7/pc42 H2 and HuA L3 (Fig. 1A). Similar results were obtained with C. albicans NCPF 3153 (data not shown). Most H2 alanine-substituted derivatives (asd) had activities similar to H2, but some showed modulating effects (Table 2). Substitution of the last residue in H2 (K16A) resulted in a fundamental loss of activity whereas substitution of the previous amino acid (F15A) gave rise to the highest candidacidal derivative.

Anti HIV-1 activity of CDR-based synthetic peptides

The ex vivo and in vitro activities of synthetic peptides corresponding to mAb C7, mAb pc42, mAb HuA CDRs and asd of mAb C7/pc42 H1 against HIV-1 are shown in Tables 3 and 4. The kinetics of viral Ag production in untreated cultures corresponded to 100% of viral production. Results are represent-
determinations observed on day 10 of cultures. In condition. Percent values of HIV-1 inhibition express the mean of 4 independent experiments performed for each assay.

Table 2. In vitro microbialicidal activity of mAb C7, mAb pc42 and mAb HuA CDRs tested as synthetic peptides against Candida albicans.

| mAb CDR | Anti-Candida activity | EC50 (95% confidence intervals) [mol/L] |
|----------|-----------------------|----------------------------------------|
| C7 L1    | KSSQSLNNSGNQK NYLT   | 100 (1.123–2.066)×10^{-5}             |
| C7 L2    | WASTRES               | 49.8                                   |
| C7 L3    | NDYSYPRSR             | 99.3 (2.120–3.206)×10^{-5}             |
| C7/pc42 H1 | GYMMH               | 69.2                                   |
| C7/pc42 H2 | YISCYNGATSYNFQFK     | 100 (3.416–5.146)×10^{-6}             |
| C7 H3    | ARQVVRGAMAID          | 55.5                                   |
| pc42 L1  | YRASKVSTGYSYMH       | 100 (7.401–8.223)×10^{-7}             |
| pc42 L2  | LVSNLES               | 100 (1.115–1.283)×10^{-5}             |
| pc42 L3  | QHIRELTRSE           | 83.9                                   |
| pc42 H3  | PNPLKAM               | 25.9                                   |
| HuA L1   | RASQVSVSYLA          | 0                                     |
| HuA L2   | DASNRAT               | 0                                     |
| HuA L3   | QORSNWPRS            | 100 (3.408–7.662)×10^{-6}             |
| HuA H1   | SYTFH                 | 12.9                                   |
| HuA H2   | VLYLDSYQHYADYSVKG    | 0                                     |
| HuA H3   | GQTVTKEIDY           | 19.2                                   |

doi:10.1371/journal.pone.0002371.t001

Table 3. Ex vivo and in vitro inhibitory activity (%) of synthetic mAb C7, mAb pc42, and mAb HuA CDRs against HIV-1.

| mAb CDR | % inhibitory activity (10 μg/ml) |
|----------|---------------------------------|
| endogenous replication | exogenous HIV-1 replication |
| HIV-1 | BaL | IIIB | HIV-1 | BaL | IIIB |
| C7 L1 | 37 | 9 | 0 |
| C7 L2 | 38 | 59 | 4 |
| C7 L3 | 49 | 18 | 0 |
| C7/pc42 H1 | 58 | 24 | 0 |
| C7/pc42 H2 | 18 | 0 | 0 |
| C7 H3 | 0 | 75 | 0 |
| pc42 L1 | 91 | 41 | 0 |
| pc42 L2 | 0 | 0 | 0 |
| pc42 L3 | 0 | 0 | 0 |
| pc42 H3 | 11 | 0 | 0 |
| HuA L1 | 48 | 15 | 0 |
| HuA L2 | 32 | 0 | 0 |
| HuA L3 | 70 | 63 | 0 |
| HuA H1 | 84 | 84 | 0 |
| HuA H2 | 40 | 74 | 0 |
| HuA H3 | 36 | 0 | 0 |

doi:10.1371/journal.pone.0002371.t003

Antimicrobial activities of CDR-based synthetic peptides

All CDR peptides from all three mAbs were assayed for cytotoxicity in B16F10-Nex2 murine melanoma cells. Although most of them were inactive, C7/pc42 H2 inhibited 50% of tumor cell growth at 0.05 mM; mAb HuA L1 was also effective but 5–10-fold less inhibitory than C7/pc42 H2 (Table 5). MAb C7/pc42 H2 was equally cytototox to human melanoma cell lines SKmel-25 and SKmel-28 (Fig. 2A). Both C7/pc42 H2 and HuA L1 caused DNA degradation in melanoma cells (Fig. 2B). The corresponding scrambled peptides were inactive. Additional data confirmed that both peptides were apoptotic not only in melanoma cells but also in HL-60 leukemia cells. Apoptosis was caspase-dependent and inhibitable by z-VAD, a pan-caspase inhibitor. Typical apoptotic alterations were seen in HL-60 cells with surface blebs and nuclear fragmentation (Fig. 2C).
Table 4. *Ex vivo* inhibitory activity (%) of synthetic mAb C7 CDR H1 alanine-substituted derivatives against HIV-1.

| mAb C7/pc42 H1 asd | % inhibitory activity (10 μg/ml) |
|---------------------|---------------------------------|
| G1A                 | 0                               |
| Y2A                 | 61                              |
| Y3A                 | 90                              |
| M4A                 | 41                              |
| H5A                 | 0                               |

*asd: alanine-substituted derivatives.

doi:10.1371/journal.pone.0002371.t004

Table 5. *In vitro* antitumor activity of selected synthetic mAb C7, mAb pc42, and mAb HuA CDRs against B16F10-Nex2 murine melanoma cells.

| CDR         | EC50 (95% confidence intervals) [mol/L] |
|-------------|----------------------------------------|
| mAb C7/pc42 H2 | 5.35 (2.220–8.480)×10^{-3}             |
| mAb HuA L1    | 7.20 (6.666–7.734)×10^{-4}             |

doi:10.1371/journal.pone.0002371.t005

C7/pc42 H2 peptide is predicted to contain a beta amphipathic (Eisenberg) region corresponding to SYNKFK C-terminal sequence. Alanine substitutions in this region, rather than on the N-terminal (YISCYN) and turn region (GAT) reduced the cytotoxicity of H2 (Table 6).

*In vivo* growth of tumor cells depends strictly on angiogenesis, therefore CDR peptides were also tested for endothelial cell cytotoxicity. The mAb C7 CDRs inhibited endothelial cell sprouting as tested in *vitro* with HUVEC cells grown on Matrigel®. CDRs H2, and also L1, L2 and L3 at higher concentrations, inhibited endothelial cell sprouting (Fig. 3A, B). These results show that CDRs from the same mAb, but mostly H2 have a capacity to inhibit both tumor cell growth and endothelial cell motility and tube formation.

Administration of mAb C7/pc42 H2 and mAb HuA L1 intraperitoneally showed antitumor effects in *vivo* in a model of lung colonization by melanoma cells. While the untreated controls had >300 black nodules in the lungs as a result of intravenous injection of B16F10-Nex2 cells syngeneic to C57BL6 mice, the peptide-treated animals had lungs with 34–40 nodules. A significant increase in the survival time of peptide-treated animals was observed; mice died several days after peptide treatment was interrupted (Fig. 4A, B, C).

**Discussion**

CDR sequences in the variable regions of immunoglobulins are thought to act cooperatively in the recognition of an antigen. Among them, CDR H3 is at the center of antigen recognition, but the other five CDRs are more or less involved for increased binding affinity to the antigen and some contact residues can even be located within framework regions [17]. The observation that Ab specificity is determined by a limited number of residues has allowed the synthesis of small peptides based on CDRs which retain binding properties and functions of the intact Ab [18,19]. Diversity of CDR1 and CDR2 is encoded by the germline and further modified by somatic mutation; that of CDR L3 and CDR H3 arises somatically by rearrangement of the V segment with the JL or DH and JH segments, respectively.

Isolated CDR sequences, frequently CDR H3, showed the same specificity of the native Ab and were called micro(mini)antibodies [20,21]. Diversity of CDR H3 is sufficient to allow otherwise identical IgM molecules to distinguish between a variety of haptens and protein antigens [22]. It has even been postulated that diverse CDR3 loops represent a highly antigen specific recognition core whereas other CDRs bind opportunistically [23]. Recently, a tyrosine-sulfated peptide derived from CDR H3 of an HIV-1-neutralizing Ab was shown to bind gp120 and inhibit HIV-1 infection [24]. Apart from the microantibodies that may display anti-viral [25] and antitumor [26] activities as does the native Ab, less is known about the biological activities of other CDR and framework sequences tested as isolated peptides. Some CDR and framework region-derived peptides, however, have been described as inhibitors of receptor-ligand interactions, cell adhesion and of microbial or viral infections [5,27]. Several peptides including amino acids from the CDRs of anti-C4d mAb ST40 and framework residues flanking the CDRs bound to soluble C4d and displaced Ab binding [28]. Bioactive paratope-derived peptides of potential pharmacological interest were also deduced by hydrophilic complementarity [29].

Here we show that, independent of the specificity of the native Ab, CDRs other than H3 may display, with high frequency, antimicrobial, antiviral and antitumor activities in a way reminiscent of molecules of early innate immunity [30]. Synthetic peptides representing the CDRs of a native Ab (mAb C7), raised against a C. albicans antigen, and also CDRs from mouse mAb pc42, sharing H1 and H2 with mAb C7, and human mAb HuA, sharing no CDR either with mAb C7 or mAb pc42, showed *in vitro*, *ex vivo* and/or *in vivo* differential antimicrobial, antiviral and/or antitumor activities.

The *in vivo* antitumor activity of mAb C7/pc42 H2 and mAb HuA L1, the protection conferred by mAb C7/pc42 H1, H2 and mAb C7 L1 against invasive candidiasis, as well as *ex vivo* inhibitory activity of mAb pc42 L1 against HIV-1 are examples of hypervariable Ab sequences with biological activity. Synthetic peptides representing Ig CDRs are linear sequences with specific binding properties. The high frequency of peptide binding in Ig CDRs may reflect their increased diversity by somatic mutation and clonal selection by antigens. Whether there may occur a proteolytic release of active fragments from immunoglobulins is a debatable hypothesis, that would be reminiscent of the extrinsic activity of Hb33-61 from bovine haemoglobin that displays antimicrobial activity against Gram-positive bacteria and fungi at μM concentrations, as well as the carboxy-terminal tripeptide (11-13, KPV) of α-MSH that inhibits *Staphylococcus aureus*, *C. albicans* and HIV-1 at picomolar concentrations [31–33].

A wide variety of organisms, from single-cell microorganisms, insects and other invertebrates, plants, amphibians, birds, fishes, and mammals, man included, produce antimicrobial, antiparasitic and antiviral peptides as part of their first line of defense [34]. As evaluated by searching the Blast (National Center for Biotechnology Information) data base, however, the sequences of the Ab CDRs investigated in this study appear to be characteristic of immunoglobulins and are very different from those of described natural peptides.

Here, it is clear that C7/pc42 H2 and HuA L1 are directly cytotoxic to tumor cells causing caspase-dependent apoptosis. Apparently, the pro-apoptotic activity of C7/pc42 H2 on melanoma cells depended on peptide binding to a surface receptor that specifically recognizes the C-terminal sequence. The C-terminal SYNKFK peptide which is not apoptotic by itself...
competed with and inhibited the cytotoxicity of mAb C7/pc42 H2 in melanoma cells (data not shown). Amino acids K14 and F15 are crucial for the receptor-mediated mAb C7/pc42 H2 tumor cell cytotoxicity. C7 H3 but not C7/pc42 H2 competed with mAb C7 for binding to phosphatidylcholine, the probable ligand of polyreactive C7 on melanoma cells (unpublished results).

Apoptosis was characterized in both melanoma and HL-60 leukemia cells. H2 also inhibited endothelial cell growth on Matrigel™ suggesting that its in vivo antitumor effect could also involve inhibition of angiogenesis. Presently we show that both peptides in the more stable C-terminal amidated form were active against lung colonization by melanoma cells injected i.v. using a protocol of i.p. administration every-other-day for 11 days. A direct effect of peptides on tumor cells was needed for protection since the suspension of treatment resulted in a delayed death curve similar to the untreated control.

CDRs interference on HIV-1 replication was studied in PBMCs exogenously infected with different viral strains and in a more physiologic model of endogenously infected mononuclear cells. Presently, we show that some CDR synthetic peptides were able to inhibit R5 HIV-1 expression in naturally and exogenously infected PBMCs. We speculate that the interaction between CDR molecules and HIV-1 co-receptors CCR5 and CXCR4 in our culture systems were, probably, the limiting factor for HIV-1 replication. An alignment analysis (http://www.ebi.ac.uk/cgi-bin/clustalw) of the viral protein with the CDR sequences with high antiviral activity showed that important peptide motifs of HuA L3 (QRSNWPR) and of pc42 L1 (SKSVSTSG) were present in
reverse transcriptase (RT) and Rev proteins, respectively. For HIV-1 inhibition, it has already been reported that many peptides could be competitive or non-competitive inhibitors of enzymatic activities by structure mimicry or by hindering the formation of enzyme-DNA/RNA complexes [35,36].

Possible molecular targets in Candida to reactive pc42 L1 and L2 and HuA L3 CDRs could be β-1,3/β-1,6 glucans that neutralize these peptides as described for KP (data not shown). In the case of C7 H2, an N-terminal sequence in ALS3 is hypothesized to be a target based on its functional similarity with a peptide deduced by hydropathic complementarity to C7 H2 with codons read in the 3’→5’ direction [37].

Additional pathogenic agents and tumor cell lines obtained from type culture collections or isolated from clinical samples were also sensitive in vitro to the inhibitory activity of mAb C7, pc42 and HuA CDRs and engineered derivatives (unpublished data). They include eukaryotic and prokaryotic microorganisms such as C. neoformans, A. fumigatus, S. prolificans, Pseudomonas aeruginosa, methicillin-resistant S. aureus, viruses such as influenza A Ulster 73 (H7N1) and human NWS (neurotropic H1N1) and tumor cell lines HeLa (human cervix epitheloid carcinoma), CEM (human leukemia), Hs294T (human melanoma).

The easy production and the low cost of small sized synthetic peptides representing Ig CDRs may offer significant advantages compared to recombinant Abs or Ab fragments for the rational identification and design of novel antitumor, antimicrobial and antiviral biologically active and therapeutic compounds. As we demonstrated with KP and its D-isomeric form, such peptides can be easily engineered and stabilized against proteolysis by incorporation of non-natural amino acids or other modifications without affecting, or even enhancing, their activity [7,38]. Peptide engineering and chemical optimization associated to new delivery mechanisms are expected to provide a new generation of therapeutic agents in parallel to the peptide vaccines that aim at protective immune responses [39].

Table 6. In vitro antitumor activity of synthetic mAb C7/pc42 H2 alanine-substituted derivatives against B16F10-Nex2 murine melanoma cells.

| mAb C7/pc42 asd | EC50 (95% confidence intervals) [mol/L] | EC50asd/EC50H2 |
|-----------------|----------------------------------------|-----------------|
| Y1A             | 7.14 (3.587–10.693)×10⁻⁵               | 1.33            |
| J1A             | 7.35 (3.847–10.853)×10⁻⁵               | 1.37            |
| S3A             | 6.49 (4.751–8.229)×10⁻⁵               | 1.21            |
| C4A             | 6.75 (3.222–10.278)×10⁻⁵               | 1.26            |
| Y5A             | 7.35 (3.773–10.927)×10⁻⁵               | 1.37            |
| N6A             | 7.69 (5.951–9.429)×10⁻⁵               | 1.44            |
| G7A             | 6.32 (2.817–9.823)×10⁻⁵               | 1.18            |
| T9A             | 6.02 (2.517–9.523)×10⁻⁵               | 1.13            |
| S10A            | 1.19 (1.016–1.364)×10⁻⁴               | 2.22            |
| Y11A            | 1.13 (0.954–1.306)×10⁻⁴               | 2.11            |
| N12A            | 1.25 (1.163–1.337)×10⁻⁴               | 2.34            |
| Q13A            | 1.28 (0.922–1.638)×10⁻⁴               | 2.39            |
| K14A            | 3.84 (3.654–4.026)×10⁻⁴               | 7.18            |
| F15A            | 1.00 (0.982–1.018)×10⁻³               | 18.69           |
| K16A            | 8.7 (5.073–12.327)×10⁻⁵               | 1.63            |

asd: alanine-substituted derivatives.
doi:10.1371/journal.pone.0002371.t006

Materials and Methods

Monoclonal antibodies and CDRs

Mouse mAb C7 (IgM), was produced as described elsewhere [11]. Single-chain variable region Ab fragments were obtained from the RNA extracted from the hybridoma cells secreting mAb C7 (scFv C7) by using the phage display methodology, as described [3]. After scFv C7 sequencing the CDRs of both light and heavy chains were identified [40]. All the CDRs of mAb C7 were chemically synthesized.

The mouse mAb pc42 (IgM), was selected from the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information data base, searching for short, nearly exact matches to the CDRs of mAb C7, as both mAbs shared the same CDRs H1 and H2. MAb pc42 is directed to the dominant epitope within a synthetic peptide model antigen (PS1CT3).
including residues 28–42 of the large protein of the surface antigen of hepatitis B virus containing a B cell epitope (PS1) and the known T-helper lymphocyte epitope derived from the circumsporozoite protein of the malaria parasite P. falciparum (CT3) [15].

The hybridoma secreting mAb pc42 was kindly provided by Dr. Kanury V.S. Rao (International Center for Genetic Engineering and Biotechnology, New Delhi, India). As only the sequence of the heavy chain with the corresponding CDRs (H1, H2, H3) had been defined and published for mAb pc42, the genes encoding its light chain were cloned and sequenced and the CDRs (L1, L2 and L3) identified according to the procedures described for mAb C7.

Human mAb HuA (IgM) was secreted by a human-mouse trioma cell line which was a cloned Epstein Barr virus-transformed B cell line stabilized by fusion with the human-mouse fusion partner SBC-H20. The V_H and V_L chain cDNAs of HuA had been sequenced [16]. This Ab is representative of Abs widely spread in the population and all its CDRs differed from those of mAb C7 and mAb pc42. MAb HuA is specific for the most frequent difucosylated human blood group A substances. All the CDRs of mAb pc42 and mAb HuA were chemically synthesized. The mAb C7/pc42 H2 and mAb HuA L1 scrambled peptides had the sequences: QYKISCNKYTGSFNY and YARQVSSALSS, respectively.

Engineering and evaluation of selected mAb C7 CDRs

The CDRs shared by mAb C7 and mAb pc42 (H1 and H2) showing antimicrobial, antiviral and/or antitumor activity in vitro or ex vivo were engineered by alanine-scanning. The alanine substituted derivatives (asd), defined according to the position held by the alanine-substituted aminoacid, were tested in the same biological assays to critically establish the functional relevance of each residue [5]. In vitro microbicidal activity of CDR peptides and selected asd against C. albicans UP 10 (clinical isolate from the fungi collection of the University of Parma) and C. albicans NCPF 3153 (National Collection of Pathogenic Fungi, Bristol, UK) were used for the in vitro experiments.

The microbicidal activity of synthetic CDRs against C. albicans strains was preliminarily evaluated by testing 100 μg/mL of each synthetic peptide by colony forming unit (CFU) assays as previously described [5]. Briefly, 10 μL of a distilled water suspension containing ~5×10^4/mL germinating yeast cells were added to 90 μL of distilled water containing the synthetic peptide to be tested. Yeast suspensions were incubated at 37°C for 6 h. After incubation, yeast suspensions were plated on Sabouraud dextrose agar, incubated at 30°C and observed for CFU determination after 48–72 hours. Each assay was carried out in triplicate. MAb CDR synthetic peptides exhibiting candidacidal activity at 100 μg/mL, and asd of mAb C7/pc42 H2, selected for the highest candidacidal activity, were further tested to determine EC50 values. EC50 was calculated by nonlinear regression analysis using Graph Pad Prism 4.01 software, San Diego, CA, USA.

In vivo evaluation of anti-Candida activity of CDR peptides

Animal experiments were approved by the Institutional Review Board of the School of Medicine and Odontology at the University of the Basque Country, Spain. Female BALB/c mice, 8-weeks old (11–12 animals/group), were infected intravenously with 5×10^7 C. albicans NCPF 3153 yeast cells suspended in 0.1 mL saline. Mice were treated i.p. with 200 μg of mAb C7 L1 and mAb C7/pc42 H1 and H2 for 3 days starting on day 0, and 4 h after the fungal challenge, and at 24 and 48 h thereafter. Control mice were injected with saline (same treatment schedule as those treated with the CDRs). Protection was evaluated by monitoring animal death.
survival for 20 days. The mean survival time and numbers of CFU of C. albicans in infected tissues were calculated as reported previously [14].

**Ex vivo and in vitro evaluation of the inhibitory activity of CDR peptides and selected asd against HIV-1**

Assessment of *ex vivo* antiviral activity of synthetic CDRs and *asd* of mAb C7 H1, selected for the highest antiviral activity in HIV-1 endogenous infection was performed as previously described [10,41]. Briefly, for endogenous HIV-1 replication assays peripheral blood mononuclear cells (PBMCs) from R5 HIV-1-infected patients characterized by high viral RNA titers in plasma and cell-associated viremia were cultured in 96-well plates at 1 × 10^5 cells/mL in RPMI 1640 medium with 10% fetal calf serum (FCS) and 20 U/mL of rIL-2. Exogenous rIL-2 was added every 3–4 days. Cultures were treated with synthetic mAb CDRs and mAb C7/pc42 H1 *asd* at 10 μg/mL during the time of culture. Viral production on days 3 and 5 was assessed in the supernatants using ultrasensitive Alliance® HIV-1 p24 ELISA kit (Perkin Elmer); p24 Ag in the supernatants of untreated cultures corresponded to 100% of viral production. For exogenous infection assays, PHA-stimulated PBMCs obtained from healthy individuals were infected with BaL (R5) or IIB (X4) HIV-1 strain at a median tissue culture infective dose (TCID_{50}) of 500 TCID_{50}/mL. After 2 h of adsorption, the cells were washed, suspended at 2 × 10^5 cells/mL in medium and cultured in 96-well culture plates. At time zero of infection, synthetic peptides were added to the cultures at 10 μg/mL and were maintained throughout the experiment. All assays were performed in triplicate. Virus production was assayed 10 days after infection in the supernatants of HIV-1-infected PBMCs by p24 assay.

**Tumor cell lines and cell culture**

Murine B16F10-Nex2 melanoma cells (cloned at the Experimental Oncology Unit, Federal University of São Paulo, UNIFESP), and human SKme128 and SKme25 human melanoma cell lines originally obtained from the Memorial Sloan Kettering Cancer Center, New York, were cultured at 37°C, under humid atmosphere and 5% CO₂, in RPMI-1640 medium with 10 mM N-2-hydroxyethylpiperazine-N2 ethanesulfonic acid (HEPES), 24 mM sodium bicarbonate, 40 mg/L gentamycin, pH 7.2 and 10% FCS.

**In vitro tumor cytotoxic activity of CDR peptides and selected asd**

Synthetic CDRs, scrambled peptides (controls), and *asd* of mAb C7/pc42 H2, selected for the highest cytotoxic activity, were diluted from 1 mM to 0.05 mM in RPMI with 10% FCS and incubated with B16F10-Nex2, SKme128 and SKme25 cells (5 × 10^3 cells/well) in 100 μL per well for 12 h at 37°C. Each peptide was tested in triplicate. After 12 h, the cytotoxic activities of the peptides were determined by measuring cell viability by Trypan Blue exclusion. A 50% inhibition of cell growth was taken as a comparative index of cytotoxicity (EC_{50}).

**DNA fragmentation assay**

B16F10 cells were grown for 24 h in 12-well plates (10^4 cells/well), and then were further incubated for 12 h at 37°C with mAb C7/pc42 H2 (0.3 mM) and mAb HuA L1 (0.6 mM). Cells were then collected in phosphate-buffered saline-EDTA (PBS-0.02% EDTA) and centrifuged at 1,500 rpm. The pellets were suspended in 500 μL of TELT buffer [42] [50 mM Tris-HCl (pH 7.4), 5% Triton X-100 (LKB), 2.5 mM EDTA, pH 9.0 (Pharmacia) and 2.5 M LiCl (Merck)], pH 8.0. After centrifugation, equal volume of phenol (Gibco, pH 7.49–7.79) was added to the supernatant and centrifuged at 12,000 g/30 minutes. The aqueous phase was transferred to a new tube and equal volume of chloroform was added (Merck). After centrifugation (15 min, 12,000 g), equal volume of isopropanol was added to the pellet and cooled at −20°C for 24 h. The sample was centrifuged at 12,000 g/15 min, and the pellet washed with 500 μL of ethanol 70% (Merck). The dry pellet was resuspended in water with RNAse (20 μg/mL). Degradation of DNA in a ladder profile [43] was assayed in 0.8% Agarose gel electrophoresis (Pharmacia).

**In vitro Matrigel angiogenesis assay**

Matrigel™ Matrix (BD Biosciences, Bedford, MA, USA) was thawed on ice, added (10 μL/well) to coat 96-well plates, and allowed to polymerize for 1 h at 37°C. Human umbilical vein endothelial cells (HUVEC) (5 × 10^4 cells/well) were added alone or mixed with mAb C7 CDR peptides in 100 μL of RPMI medium supplemented with 0.2% of FCS in each well. All peptides were used at 1 mM, with the exception of mAb C7/pc42 H2 that was used at 50 μM. The plates were incubated at 37°C for 18 h and images were captured at 8× magnification with a Sony Cyber-shot camera coupled to a light microscope. The number of pro-angiogenic structures (closed rings formed at a given time by endothelial cell sprouting) was counted from 3 different wells, and the average value determined in each system [44,45]. The assay was repeated at least three times.

**In vivo tumor protection assays with CDR peptides**

All animal experiments were carried out using protocols approved by the Ethics Committee for animal experimentation of Federal University of São Paulo, Brazil. Thirty male C57BL/6 mice seven-to eight-week-old were injected i.v. with 5 × 10^5 syngeneic B16F10-Nex2 viable cells in 0.1 mL for each mouse. For protection experiments three groups of 10 animals received on days 1, 3, 5, 7, 9, 11 after tumor cell challenge, i.p. doses of 250 μg of mAb C7/pc42 H2 and of mAb HuA L1, or PBS, at the same time periods, respectively. After 23 days, the lungs were collected from half of the animals of each group and inspected for metastatic colonization and the melanotic nodules were counted at 2× magnification. The survivals of remaining peptide-treated mice and controls were recorded till 40 days after i.v. challenge of tumor cells.

**Statistical analysis**

The Kaplan-Meier log rank test was applied to survival data. Data on CFU in infected tissues were analyzed by Student’s *t* test. *p* values of 0.05 were considered significant.

**Acknowledgments**

The hybridoma secreting mAb pc42 was kindly provided by Dr. Kanury Rao (Immunology Group, International Center for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi, India). HL-60 human leukemia cell line was provided by Dr. Gustavo A. Mendes, University of São Paulo.

**Author Contributions**

Conceived and designed the experiments: LT, LP, JP, CC EP PR, AD, ER DM WM SC. Performed the experiments: NE MM CC EP PR AD ER DM WM SC. Analyzed the data: LT, LP, NE MM CC EP PR AD ER DM WM SC. Contributed reagents/materials/analysis tools: MM MJ. Wrote the paper: LT, LP, JP.
References

1. Polonnell L, Lorenzini R, De Bernardi F, Gerolmi M, Coni S, et al. (1993) Idiotypic vaccination: Immunoprotection mediated by idiotypic antibodies with antitumoric activity. Scand J Immunol 37: 105–110.

2. Polonnell L, De Bernardi F, Coni S, Boccarda M, Magliani W, et al. (1996) Human natural yeast killer toxin-like candidalactid antibodies. J Immunol 156: 1889–1893.

3. Magliani W, Coni S, De Bernardi F, Gerolmi M, Bertolotti D, et al. (1997) Therapeutic potential of an antitoxin single chain antibodies with yeast killer toxin activity. Nat Biotechnol 15: 155–158.

4. Cenci E, Menacace A, Spreca A, Montagnoli C, Bacci A, et al. (2002) Protection of killer antitoxic antibodies against early invasive aspergillosis in a murine model of allogeneic T-cell-depleted bone marrow transplantation. Infect Immun 70: 2375–2382.

5. Polonnell L, Magliani W, Coni S, Bracci L, Lozzi L, et al. (2003) Therapeutic activity of an engineered synthetic killer antitoxic antibody fragment against experimental mucosal and systemic candidiasis. Infect Immun 71: 6205–6212.

6. Cenci E, Bistoni F, Menacace A, Perito S, Magliani W, et al. (2004) A synthetic peptide as a novel antitoxic tool against K562. Cell Microbial 6: 953–961.

7. Travassos LR, Silva LS, Rodrigues EG, Coni S, Salati A, et al. (2004) Therapeutic activity of a killer peptide against experimental paracoccidiodomycosis. J Antimicrob Chemother 54: 956–958.

8. Coni S, Fanti F, Magliani W, Gerolmi M, Bertolotti D, et al. (1998) Mycobacterial activity of human natural, monoclonal, and recombinant yeast killer-toxin-like antibodies. J Infect Dis 177: 807–811.

9. Fiori PL, Mattana A, Desi D, Coni S, Magliani W, et al. (2006) In vitro antacanthamoebial activity of a killer monoclonal antibody and a synthetic peptide. J Antimicrob Chemother 57: 891–898.

10. Casoli C, Pilotti E, Perno CF, Balestra E, Polverini E, et al. (2006) A killer protein with therapeutic activity against AIDS-related opportunistic microorganisms inhibits extrinsic HIV-1 replication. AIDS 20: 975–980.

11. Moragues MD, Omaetxebarria MJ, Elguezabal N, Coni S, Gutiérrez A, et al. (2003) A monoclonal antibody directed against a Candida albicans cell wall mannanprotein exerts three anti-C. albicans activities. Infect Immun 71: 5273–5279.

12. Omaetxebarria MJ, Moragues MD, Elguzabal N, Rodríguez-Alejandre A, Brenna S, et al. (2005) Antifungal and antitumor activities of a monoclonal antibody directed against a stress mannanprotein of Candida albicans. Curr Med Chem 5: 393–401.

13. Brenn S, Omaetxebarria MJ, Elguzabal N, Cabezas J, Moragues MD, et al. (2007) The fungal monoclonal antibody G7 binds to Candida albicans Ak3. Infect Immun 75: 3680–3682.

14. Sevilla MJ, Rohleder B, Rementeria A, Moragues MD, Pontón J (2006) A fungal monoclonal antibody protects against murine invasive candidiasis. Infect Immun 74: 3042–3045.

15. Tuteja R (1999) B-cell responses to a peptide epitope: mutations in heavy-chain complementarity-determining region derived from a neutralizing monoclonal antibody. J Antimicrob Chemother 57: 891–898.

16. Nickerson KG, Tao MH, Chen HT, Larrick J, Kabat EA (1995) Human and murine monoclonal antibodies to blood-group A substance, which are nearly identical immunochemically, use radically different primary sequences. J Biol Chem 270: 12457–12463.

17. Davies DR, Cohen GH (1996) Interactions of protein antigens with antibodies. Proc Nat Acad Sci USA 93: 7–12.

18. Padlan EA, Aboriginal W, Tipper JP (1995) Identification of specificity-determining residues in antibodies. Faseb J 9: 133–139.

19. Eisenhardt SE, Schwartz M, Schaller N, Sooarajiah J, Basler N, et al. (2007) Generation of activation-specific human anti-alphaMbeta2 single-chain antibodies as potential diagnostic tools and therapeutic agents. Blood 109: 3521–3528.

20. Levi M, Salberg M, Ruder U, Herlyn D, Maruyama H, et al. (1993) A complementarity-determining region synthetic peptide acts as a mimotaxibody and neutralizes human-immunodeficiency-virus type-1 in vitro. Proc Nat Acad Sci USA 90: 4374–4378.

21. Bourgeois C, Bour JB, Aho LS, Pothier P (1998) Prophylactic administration of a synthetic anti-HER2/neu peptide mimetic disables P143HER2/neu tyrosine kinases in vitro and in vivo. Nat Biotechnol 18: 194–196.

22. Feng Y, Chung D, Garrard L, McEneary G, Lim D, et al. (1998) Peptides derived from the complementarity-determining regions of anti-Mac-1 antibodies block intercellular adhesion molecule-1 interaction with Mac-1. J Biol Chem 273: 5625–5630.

23. Davis MM (2004) The evolutionary and structural ‘logic’ of antigen receptor diversity. Semin Immunol 16: 239–243.