CHAPTER 21

FUNGAL VACCINES AND VACCINATION: PROBLEMS AND PERSPECTIVES

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Abstract: Vaccines against human pathogenic fungi, a rather neglected medical need until few years ago, are now gaining steps in the public health priority scale. The awareness of the rising medical threat represented by the opportunistic fungal infections among the health care-associated infections, the advances in the knowledge of fungal pathogenicity and immune response and the extraordinary progress of biotechnology have generated enthusiasm and critical new tools for active and passive anti-fungal vaccination. The discovery that antibodies play a critical role for protection against fungal infection has greatly contributed to the advancements in this field, in recognition that almost all useful vaccines against viral and bacterial pathogens owe their protective efficacy to neutralizing, opsonizing or otherwise effective antibodies. Overall, there is more hope now than few years ago about the chances of generating and having approved by the regulatory authorities one or more antifungal vaccines, be active or passive, for use in humans in the next few years. In particular, the possibility of protecting against multiple opportunistic mycoses in immuno-depressed subjects with a single, well-defined glucan-conjugate vaccine eliciting directly anti-fungal antibodies may be an important step to achieve this public health goal

1. INTRODUCTION

The vaccines represent the most useful immunological application for human health. They are the only medical tools that, when used prophylactically, allow disease elimination or even eradication of the causative agent, as has happened with smallpox and is hopefully close to occur with poliomyelitis. When disease elimination is, for various reasons, bound to the habitat of the infecting organism and the natural history of infection, impossible or unlikely, yet the availability of a protective vaccine and effective vaccination procedures results into an effective control of the disease as witnessed for diseases such hepatitis B, measles and pertussis, just to cite only a few. To reach these goals in terms of public health, ranking

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from disease eradication to elimination and control, the availability of a safe and efficacious vaccine is obviously essential. However, critical factors are also the definition of the medical need of any given vaccine in terms of cost/benefit, the prospected advantage in relation to the existing preventive or therapeutic measures, the correct identification of the target population to vaccinate. The capability of the public health system to implement an effective vaccination policy must also be carefully considered. This includes, among others, the capacity to persuade healthy subjects or their parents or guardians in case of pediatric vaccines, of the personal and societal advantages of being vaccinated, weighting the minimal though actual risk of an adverse event against all future benefits of avoiding the diseases and its transmission to neighbours. Finally, of utmost importance is also establishing whether the disease could be contrasted not only with a preventive vaccination but also with the use of a therapeutic vaccine, i.e. a vaccine formulation that could be used in the ill subject. This is an exciting, very attractive approach which deserves particular attention in the field of fungal diseases. However, no therapeutic vaccine successfully used to fight infection has been so far provided.

All the elements above are balanced in the decision of undertaking vaccine manufacturing and establishing vaccination priority. This becomes particularly cogent in the case of diseases for which either the true incidence and impact on public health are unknown or the identification of the target population is problematic. Fungal diseases of humans are exactly a case in point. Besides few severe but geographically-limited and relatively low-incidence deep-seated infections such as, for instance, coccidiomycosis and blastomycosis, most other incident worldwide infections such as aspergillosis, cryptococcosis and candidiasis (in this last case, with the possible exception of the vaginal candidiasis) typically occur in the immunocompromised host, a clinical setting which raises remarkable obstacles to the rationale itself of the immunopreventive or even therapeutic vaccination. This is mostly due to difficulties in achieving an efficient and long-lasting immune response, in identifying who could really benefit of the vaccine, and establishing how and when exactly to vaccinate. Thus, it doesn't make a surprise that fungal vaccines have been unfairly placed in a rather remote room in the public health building, as witnessed by the practical absence of information on antifungal vaccines in one renowned, most famous textbook on Vaccines (Plotkin, and Orenstein, 1999), or in special editorial overviews,with only few lines of description even in the Jordan Report (2004) the most detailed state of art document annually released by the National Health Institutes.

This being said, there are several reasons and evidence gathered in the last years commanding the scale up of fungal vaccines for humans on a rather high position in the priority scale. Strong advocacy is the recent "call to arms of the immune system" launched by Stevens (2004) claiming for a vaccine against aspergillosis, a fungal disease which nobody was seriously thinking about a vaccine against until few years ago, as well as the upsurge of publications in the field of Candida vaccine (see Mochon and Cutler, 2005). It is my intention here, first, to discuss the evidence for the above command and, second, to illustrate some recent advances in the field

of fungal vaccines which makes it rather optimistic the achievement of at least a couple of effective vaccine in the next few years. I will finally focus on some novelties and conceptual advances coming from the area of fungal vaccines which could be applicable to vaccination against other human pathogens, as represented by the killer-toxin mimicking idiotypic vaccine and the ß-glucan conjugate vaccine.

Conversely, no attempt will be made here to cover all previously published findings in this area, and no details about the many different vaccine candidates and adjuvant proposed until now against the various fungal diseases will be given. Nonetheless, the most relevant information on proposed vaccine antigens and the respective references to the principal agents of fungal infections in humans are given in Table 1, while Table 2 offers indications about the most promising vaccine candidates against Candida, a rapidly growing field not only for the number of these antigens but also for the discovery of protective antibodies and their mechanism of protection (Mochon and Cutler, 2005). Moreover, mentions will be made throughout of some of the most recently proposed protective fungal antigens when their activity may account for a proposal about the immunology of protection. Finally,

Table 1. Major antigen components suggested as candidate vaccine against fungal infections (for Candidiasis, see Table 2)

Fungal disesases	Antigen	Some References
Cryptococcosis	Capsular GXM Mannoproteins Other antigens	Casadevall and Pirofski,2006; Maitta et al. 2004; Oscarsson et al. 2005
Pneumocystosis	Major surface glycopotein (gPa) p55 antigen Kexin	Smulian et al. 2000;Zheng et al. 2005
Histoplasmosis	Ribosomal proteins, Cell wall proteins, HSP60	Deepe 2004; Deepe et al. 2005;
Parococcidioidomycosis	43 Dal glycoprotein (gP43) and related multimeric peptides	Taborda et al. 2004
Blastomycosis	Blastomyces adhesin (BAD-1/or W 1–1)	Deepe et al. 2005; Wutric et al. 2005
Coccidioidomycosis	Whole cells (attenuaded or inactivated), Spherule and spherule outer wall extracts 27k-antigen Ag2/PRA Urease, HSP60	Cox and Magee, 2004; Tarcha et al. 2006.
Aspergillosis	Whole inactivated cells and crude extracts, Live attenuated conidia, Asp f 16 protease, KT-neutralizing antibody	Feldmesser, 2005; Stevens, 2004; Bozza et al. 2004

Table 2. Proposed Candida vaccines

Vaccine	Protection Mucosal; Systemic	Nature of protective immunity
Attenuated Candida strain (CA2)	−; ++	CMI, Th1
Beta −1-2 mannan	++; ++	Opsonic Abs
Ribosomal vaccine	?; +	Undefined
KT-mimicking, IdAb	++, ++	Fungicidal Abs
Inactivated whole cells (IWC)	?; +	Undefined, CMI?
Mannan-deprived IWC	?; ++	IgM Abs
Sap2	++; +?	IgG/IgA
HSP90	?; ++	Abs
P43 B cell mitogen	?; +	Abs
Ag-loaded Dendritic Cells	?; +	Th1 CMI

 ?: doubtful or not tested
 −: virulent in the mucosal model
 +: moderate protection; needs confirmation
++: strong protection
    For References, see text.

no veterinary vaccines against dermatophytes will be dealt with here. For those who wish to go into more details in all the above aspects, I advise to read a number of excellent reviews and expert opinions published by leaders in this area (just to quote a few: Deepe, 1997 and 2004; Deepe et al. 2005; Casadevall et al., 2002; Cox and Magee, 2004; Feldmesser, 2005).

2.   WHY FUNGAL VACCINES

2.1.   Incidence of Fungal Diseases

The first reason supporting the need of generating a fungal vaccine is that, overall, the prevalence and incidence of fungal infections has markedly increased worldwide, with special concern for opportunistic agents of infection. With restriction to two most frequent among the latter such as Aspergillus and Candida spp reasonable estimates suggest that at least 5% of all hospital infections are caused by these two fungi, with a specifically attributable mortality between 30 and 40% of all cases. In Italy, the estimate incidence of nosocomial infections varies from 350,000 to 400,000 new cases per year. This means that, as a minimum, between 6'000 and 8,000 subjects each year die of invasive fungal infection, a figure much larger than the actual death rate for AIDS in the same country (Urciuoli et al. 2004). These figures are rather similar in all other industrialized countries where organ transplant, invasive surgery and the plethora of all other medical conditions predisposing to fungal infection are similar. In a recent survey, Australian investigators have reported an extra 15,000 cases of aspergillosis and candidiasis per year occurring in hospitalized patients in their country (Slavin et al. 2004). In some particular at risk subjects such as the bone marrow transplanted subjects, the mortality associate with

aspergillosis may reach a figure as high as greater than 50% (Sheppard and Edwards, 2004). A recent report from the Institute of Medicine in US has emphasized that nosocomial infections are a big concern- in that they are associated with thousands of deaths each year, leading to much longer hospital stays and of course tremendous cost increase. i.e. billions of dollars in additional hospital cost each year. For aspergillosis, Stevens (2004) has reported that nearly 2'000 deaths occurred in 1996 in US, with associated 176,300 hospital days and 633 millions USD in cost. Estimates for Candida incidence mostly derive from those diseases which can be more easily diagnosed such as bloodstream and mucosal infections, particularly candidal vaginitis. Overall, this fungus has become the fourth most common agent of bloodstream infections in carefully monitored surveillance programs, with close to 10% rate respect to all other agents of bloodstream infections, and rising incidence on non-albicans species, which are intrinsically refractory to one or more antifungals (Sims et al. 2005). This fungal agent has also become a very common urinary isolate, although in this case its etiological role is much less clear. On the other side, mucosal infections by Candida have long been known as being very common, not only in immunocompromized subjects but also in apparently normal ones. A specific case here is the vaginal thrush which, in its recurrent, chronic forms, affects a substantial percentage (3 to 5%) of all women who have suffered an initial acute attack of the disease (Fidel and Sobel, 1998). Most of these infections may irreversibly affect the quality of life since they require chronic, hardly compliable antimycotic treatment and with enhanced risk of acquiring antibiotic resistance.

The focus on the most common agents of opportunistic fungal infections worldwide should not be considered a disregard of other agents of systemic fungal infections which are endemic in one or other parts of the different continents. Examples here are blastomycosis, coccidioidomycosis and paracoccidioidomycosis for which the interest in developing a vaccine remains quite high, particularly for a vaccine against Coccidioides species, despite their geographic limitation and the limited number of subjects who get sick following infection by soil spores. The questions here are, on one side, an imperfect knowledge as to whether their incidence is rising or declining and, on the other side, the difficulties to establish treatments capable of eliminating any focus of dormant cells, potentially constituting a lifelong reactivation threat.

2.2.   How Fungal Infections are Actually Treated

In contrast to most bacterial infections which can be effectively diagnosed and, despite the strong concerns about the spread of antibiotic resistance, may still be largely cured with antibiotics, fungal infections enjoy relatively few effective treatments and, for some of them, remarkable diagnostic difficulties. Some of the drugs used for combating these infections are also endowed with rather serious side-effects. The reasons for the historical paucity of efficacious antifungal treatments basically reside in the difficulties of finding suitable targets for selective drug toxicity in a microrganism which is eukaryotic as its host, coupled with some delay

by the pharmaceutical industries in investing for drugs with a limited market, as compared to bacterial and viral infections. While this gap has been partially filled in the last ten years, particularly with the novel class of echinocandins inhibiting glucan synthesis (Polak, 2003), it has also become apparent that antifungal drug resistance is not such a sporadic or episodic phenomenon as suspected until few years ago, and may rise in parallel with more widespread use of new drugs (Sanglard, 2002). The whole scenario wants either the availability of one or very few drugs, as for instance those active against some endemic mycoses, or the existence of multiple potential treatments but with the inevitable association of antibiotic resistance, as for instance in chronic mucosal candidiasis.

   As mentioned above, opportunistic invasive infections by fungi also suffer from remarkable diagnostic difficulties. For instance, invasive candidiasis is diagnosed ante-mortem in less than 50% of the patients, a diagnostic insufficiency which makes the therapeutic treatment with antifungals both delayed and often inappropriate. All this truly constitutes a sound rationale for the development of immunological preventive or therapeutic approaches.

2.3.   Vaccine Target

For endemic fungal infections which occur in otherwise healthy subjects and for which either the therapeutic options are limited or a trend toward antifungal drug resistance emerges, possibly associated with difficulties for a prompt, specific and sensitive diagnosis, the question of the vaccine target is simply answered: potentially all population groups in a defined geographic area with given characteristics, both genetic or occupational, placing them at risk of disease, in relation to the cost of any other non-specific intervention (Cox and Magee, 2004). This may also apply to other subjects which are at risk of developing fungal infections because of predisposing underlying conditions such as diabetes or invasive surgery or other local factors, but are not systemically immune-depressed to such an extent as to make vaccination unlikely to raise the correct protective immune response. Here the cost-benefit ratio is of the paramount importance as is the accurate information about the prevalence of the disease, both factors being unfortunately in most cases unknown.

   Much more complex is the identification of the target population in subjects with deep immunodepression either of the natural immunity, as for instance, the neutropenic subjects undergoing conditional chemotherapy for and after bone marrow transplantation, or of the adaptive immunity, as for primary or acquired T cell deficiency as in AIDS. Clearly these subjects are unlikely to respond protectively to vaccination owing to the partial or total lack of immune-competence, rather, they may suffer from aggravation of the immunological disorder following the immunostimulation by vaccine antigens and adjuvants. Here a crucial question is the definition of risk criteria which are associated to the likelihood of becoming ill and identify exactly the population with higher risks which could justify the cost and the possible side-effects of a vaccination before becoming immunosuppressed. While the discussion is very active in this area, there are several indications

that a rather high number of subjects at risk could indeed benefit of a advance active immunization against Candida and Aspergillus These may also include, for instance, patients candidate to transplant and those affected by tumours the therapy of which predisposes to fungal infection (Stevens 2004; Sheppard et al. 2004). Moreover, opportunistic agents of diseases have low-penetrance virulence traits and the immune responses which contribute to their control are usually redundant and impinging on both natural and adaptive immunity. Thus, a vaccine which simply potentiates a residual setting of the immunity may nonetheless be beneficial. A particular case in point here is the observation that CD8 cell activation can replace CD4 in the induction of protection against histoplasmosis in a model of CD4 cell deficient mouse, as well as the report about the direct anticandidal and anticryptococcal activity of cytotoxic CD8 T cells (Deepe, 2004; Levitz et al. 1995). Clearly, the issue is here the knowledge of the type of immune responses which help the host to rid a transmissible agent or to control a commensal fungus. This is probably the most critical aspect in the definition of a priority for an antifungal vaccine aimed to combat opportunistic fungal infections.

2.4.   Cell-Mediated and Antibody Responses: Which to Rely Upon for Vaccine-Induced Protection?

There has been a considerable debate on whether cell-mediated or antibody response is the protective arm of the antifungal immunity. Put in these terms, it is a false question, since there is no doubt that induction and fine regulation of CMI, particularly T-helper type 1 response, is a core factor in antifungal, and in general, antimicrobial response. Since an effective vaccine formulation requires induction and persistence of a protective memory response, CMI elicitation is a non-dispensable prerequisite for a valid vaccine. The question could rather be: are the cellular or the antibody effectors of immunity mostly involved in antifungal protection? These question remains of relevance for vaccination since the approach for generating a vaccine which must induce persistently activated cellular effectors, thus sustaining elevated activity of the pro-inflammatory Il-12-IFN-γ Th1 axis, may be quite different from the one which relies on B memory cells and antibody immunity, a fact which becomes still more important in partially or totally immunodepressed subjects. Thus, nature of immunizing antigen and its immunodominant epitopes, interaction with antigen presenting cells (mostly the dendritic cells) and processing through MHC class II or MHC class I pathway, and type of adjuvant, all determine the nature of elicited immunity and its outcome in terms of protection.

Most of the support for CMI effectors being the main arm of protection comes from clinical observations and well-defined animal models showing, for instance, that abolition of CD4$^+$ T cells or genetic knock-out of the Th1 cytokine pattern greatly enhances susceptibility to experimental infection by several fungi, both the dimorphic, endemic pathogens and the opportunistic ones. In addition, adoptive transfer of T cells from immunized animals has been shown to confer protection to naive counterpart in both mucosal and systemic models of infection (Romani,

2004; Santoni et al. 2002). In *C.albicans*, recombinant IFN-gamma is protective and expectedly, knock-out IFN-gamma animals are highly susceptible to infection (Romani, 2004). In humans, the situation varies with the nature of the specific infecting fungus. For instance, CMI defects, innate or acquired, predispose to severe forms of mucocutaneous but not systemic candidiasis or aspergillosis, which rather recognize neutropenia as the main predisposing condition. In contrast, crypto-coccal meningo-encephalitis, pneumonia by *Pneumocystis carinii* and deep-seated infections by dimorphic fungi such as hystoplasmosis and coccidiodomycosis are clearly favoured by the CMI defects typical of AIDS subjects. Clearly, fungi-cidal neutrophils and macrophages may better do their job when activated by Th1 cytokines such as Interferon-gamma or type 1 Interferon and TNF-alfa. We have also mentioned above the few cases where a direct anti-fungal activity by cytotoxic effector T cells has been detected in ex-vivo experiments, though the in vivo relevance of these observations remains to be established. It should not be forgotten that type 1 cytokine response is critically requested for the formation of some, highly protective antibodies against protein and most polysaccharide antigens, (e.g. IgG2a), not differently from other Th2- dependent antibodies such as IgG1 and others. Thus, the observation that CMI deletion or modification enhances diseases is not per se a definitive proof that cellular effectors of the protection are eventually involved. Moreover, various antibodies have been generated which are evidently protective in the same animal models in which cellular CMI effectors are elicited and advocated to be responsible for protection (Casadevall et al. 2002), suggesting that CMI induction eventually regulated, or was accompanied by, generation of protective antibodies. Even in one of the most evident case for a critical role of CMI effectors in controlling the infection such as the coccidioidomycosis (Cox and Magee, 2004), a recent report on a candidate vaccine antigen describes the generation of potentially protective antibodies (Tarcha et al. 2005).

DNA vaccines are believed to be the strongest immunization approach for CD4 and CD8 cytotoxic effector generation, owing to preferential antigen processing through MHC class I pathway. Nonetheless, a DNA vaccine using the Pneumocystis gene coding for kexin, a furin-like protease, and CD40 ligand as adjuvant has been recently shown to generate anti-*P.carinii* protective antibodies both in CD4-depleted and CD4 repleted mice (Zheng et al. 2005). In another approach to a vaccine against pneumocystosis with the use of the cell wall-associated, glutamic acid-repeat-rich protein of 414 amino acids (p55), Smulian et al (2000) demonstrated that partial protection from rat pneumonia was accompanied by both CMI and antibody responses. Finally, antibodies against the major surface glycoprotein (gpA) of the above fungus were manifestly protective independently on the presence of T cells (Harmsen et al. 1995). Remarkably here, pneumocystosis clinically is a major example of disease caused by CD4 T cell deficiency as in AIDS.

Three other considerations would speak against CMI effector cells being uniquely or predominantly exploited for vaccine protection: 1. Maintaining a persistent-activation of CMI effectors, that is usually acquired by whole cell or DNA vaccines, while positively controlling the infectious agent, may be nonetheless inducing strong

inflammation with potential untoward effects. Classical is the Koch phenomenon in the case of tuberculosis and this may more easily happen with some endemic dimorphic fungi, like Coccidioides which show reactivation disease (Cox and Magee, 2004). This is so true that the immune system has evolved potent means to regulate inflammation and hyper-activation of CMI and its cellular effectors through regulatory cells and counteracting cytokines. 2. Practically all bacterial and viral vaccines which have been successfully used so far owe their protective effects to antibodies, particularly toxin-neutralizing antibodies (Plotkin and Orenstein, 1999). Vaccines conceived to stimulate CMI responses with cytotoxic effectors and the accompanying array of cito- and chemokines, thus mimicking what is considered to be the protective immune response, as in the cases of the HIV and the new anti-TB vaccines, are proving extremely difficult to be achieved, despite strong efforts and investments. Recently Deepe et al. (2005), while contending that T cells, not antibodies, are the chief mediators of protective immunity against blastomycosis and histoplasmosis, also emphasized the remarkable difficulties in generating this type of vaccines; 3.Theoretically, memory-bound antibodies can be induced by vaccination in at-risk subjects before they become immunosuppressed. Because of the relative longevity of IgG and IgA, their persistence at a good protective titer in serum and mucosal secretions even during a relatively prolonged immunosuppression period is more than likely. There are several examples that this approach may work, one of the last being the protection achieved against pneumocystosis in cortisone-treated rats following vaccination with the p55 antigen or vaccination with kexin and CD40 ligand, in a DNA format, in $CD4^+$-depleted mice (Smulian et al. 2000; Zheng et al. 2005) This is clearly not achievable with vaccines merely eliciting antifungal T cells and activating macrophages or neutrophils.

2.5.   Do Antibodies Contribute to the Antifungal Protection?

If the role of CMI does not rule out antibody participating in antifungal protection, what is the "positive" evidence that antibodies do indeed have a role in this? The clinical evidence that antibodies are protective against fungal infections is rather limited to few cases (Mathews et al. 2003; Mathews and Burnie, 2004) but, in recent years, a rather strong evidence has been accumulated about the protective role of some antibodies in experimental models, some of which are close to the human disease and directly related to the use of various vaccine formulation (Tables 1 and 2). Also, thanks to the pioneering studies by Arturo Casadevall and his colleagues at Albert Einstein College of Medicine, in New York, there is now some convincing explanation of the difficulties in obtaining clinical evidence for antibody role in protection. Both for *C.neoformans* and, more recently, for *C.albicans*, this appears to be attributable to the existence of inhibitory antibodies, rather than the absence of protective ones (Casadevall, 1995; Torres et al. 2005; Bromuro et al. 2002) This situation is likely to be present in other fungal infections, both in the human- commensal or the environmental ones, and may explain the variable and inconsistent results obtained with many fungal whole cell vaccines. In turn,

the existence of inhibitory antibodies makes a new hurdle for the generation of a subunit vaccine based on antibody–mediated protection, in that it requires a critical definition and discrimination of an antigen preparation which does exclusively stimulate the production of protective antibodies, not only for their specificity but also for their isotype (Casadevall, 1995).

Meanwhile, the demonstration of protective antifungal antibodies has opened the way to the use of them as immunoprophylactic or immunotherapeutic agents against some fungi, i.e. to the feasibility of passive vaccination, an intervention which has several prospective advantages, thus deserving great attention in the area of fungal infections. Use of T cell lines and primed dendritic cells for adoptive vaccination remains the counterpart on the side of CMI-inducing vaccines (Bozza et al., 2004; Feldmesser, 2005).

3.   PASSIVE VERSUS ACTIVE VACCINATION

Historically, therapy of infection with immune sera preceded the use of both vaccines and antibiotics. Indeed, therapeutic or prophylactic antisera against diphtheria, meningitis and pneumonia, just to cite few of them, have been used soon after, or even preceding, the discovery of the causative agent of infection. The practice of passive immunization was largely abandoned not because the sera were non protective, (they were highly protective, indeed) but simply because they were either non available in a sufficient quantity or too toxic, or even caused the induction of a sometimes lethal hypersensitivity reaction to foreign proteins (serum sickness). The entry of antibiotics into the scene also contributed to push serum therapy into a corner. Nonetheless, passive vaccination has remained a viable medical approach by the use of standard or hyperimmune human immunoglobulin preparations for both pre- and post-exposure prophylaxis of diseases such as viral hepatitis, measles, varicella, tetanus and rabies. Limited specificity and limited supply are of course major disadvantages of these preparations, together with the risk of transmitting to the recipients unrecognized or undetected infectious agents (virus, prions).

It is quite clear that the present-day recombinant DNA technology is going to substantially replace the foreign sera and human immunoglobulin preparations with highly-specific humanized or human antibodies, in a variety of different formats (Traggiai et al. 2004). This has already occurred in the field of tumor and chronic, autoimmune diseases, and is taking place also in the field of infection, though to a slower rate than necessary (see for instance, Beninati et al. 2000 and Zhang et al. 2006). There are several examples of recombinant antibodies against fungal infection, and one of them is in the regulatory approval track (Mycograb, Mathews and Burnie, 2003) while others are ready to enter that path. Interestingly, a number of them are devoid of Fc component, suggesting that they can work efficiently even in the absence of phagocytic effectors cells or complement. Other monoclonal antibodies against Candida do indeed need the Fc component and a pattern of complement activation and deposition on cell surface for protection (Casadevall et al. 2002; Mochon and Cutler, 2005). So far no consistent evidence of a therapeutic

effect of passive vaccination has been provided for endemic dimorphic infections caused by Histoplasma, Coccidioides and Blastomyces, agents of typical diseases for which cellular effectors activated by the Th1 cytokines are advocated for protection. However, initial findings about protective antibodies are emerging (Deepe, 2004; Tarcha et al. 2006).

The fact that therapeutic antibodies can be generated in a human format not requiring the presence of the Fc component has particularly important implications for passive vaccination against opportunistic fungi. In theory, these antibodies can work without the cooperation of the immune system, also in such heavily immunocompromized patients, as the leukopenic ones, i.e. in the true setting of the majority of patients with deep-seated fungal infections. Because of the quantity and costs inherent in a pure antibody approach, it is more likely that antibody therapy will be used to synergize with effective antimycotics, as suggested by Mathews and Burnie in the case of Mycograb. Rather striking examples of this application have been recently provided, indirectly indicating previously undisclosed capacity of some proteins to work as protective antigens in both systemic and mucosal infection models (for instance, Sap2 and MP65 of *C.albicans*; De Bernardis et al. 2006).

4.   VACCINES AND ANTIBODIES

Having recognized that antibodies may be relevant for the control of fungal infections, thus possibly correlating with protective vaccination, a reflection is needed on the most desirable function that must have the vaccination-induced antibodies. It has already been mentioned that opsonization and complement deposition are considered of utmost importance for an effective protection, both in active and passive vaccination models. However, these antibodies require the presence of cellular effectors to fully exert their activity, a fact which can ultimately be a strong limitation in their function in immune-suppressed patients. Other antibodies have been generated following vaccination which owe their activity to the inhibition of some form of toxicity or enzyme activity (De Bernardis, 2002). Of high relevance are also other antibodies which counteract their cognate adhesins, well recognized virulence traits of several fungi (Calderone and Fonzi, 2002; Latgè and Calderone, 2002). Possibly through this inhibition, biofilm formation is affected, being biofilms critical factors for fungal growth and disease induction. That this mechanisms could be relevant for a vaccine expected to generate protective anti-fungal antibodies has recently been shown in *Candida albicans* where dual-targeting anti-adhesin domain antibodies, i.e the smallest (MW around 12,000), genetically-engineered antibody fragments containing the three complementary-determining,antigen-binding regions, exerted high level of protection both in mucosal and systemic rodent candidiasis, (De Bernardis et al. 2006). An indirect demonstration of the above mechanism has also been provided for *C.neoformans* where selected protective, but not non-protective antibodies, decreased biofilm formation in vitro (Martinez, 2005). Both in Candida and in Aspergillus, antibodies which inhibit fungal growth and even kill growing fungal cells have been described. Some of these antibodies have been

generated through vaccination with either a monoclonal antibody neutralizing a wide-spectrum, antimicrobial killer toxin and generating internal images of the toxin (the so called "idiotypic vaccination": see Polonelli et al. 1998; Cassone et al. 1997) or by immunization with stress mannoproteins, as shown by Moragues et al. 2003. More recently, a glycoconjugate vaccine composed of beta-glucan molecule, laminarin, and a diphtheria toxoid CRM197) already used as carrier protein in other bacterial vaccines, has been shown to generate fungus growth inhibitory antibodies (Torosantucci et al. 2005). This last approach warrants some specific description not only for its original immunological mechanisms of protection, i.e. direct antifungal effect not apparently relying upon host immune cooperation, but also for their potential to represent multi-target antifungal vaccines with a single preparation (cross-species immunization). If further studies will confirm the supposed identification of the killer toxin receptor in a ß-glucan molecule, the idiotypic vaccination quoted above may fall within the same kind of approach (Cassone et al. 1997).

5.   MULTITARGET ANTIFUNGAL VACCINES AND CROSS-SPECIES IMMUNIZATION

Usually, vaccines prepared to fight a given disease are made by the whole attenuated or inactivated causative microbial agent, or one or more of its immunodominant antigenic components (the so-called subunit vaccines, see Tables 1 and 2). In the nowadays very popular mixed or combined pediatric vaccines, which are aimed to immunize simultaneously against multiple diseases such as, for instance, tetanus, diphtheria, polio, pertussis and hepatitis B, a mixture of antigens from each causative agent is used. In few cases, the vaccine is composed of related, antigenically cross-reactive strain, belonging to the same bacterial or viral species, such as, for instance, in the case of the antituberculous BCG and the smallpox vaccines. To our knowledge, there is no example in the literature that a single defined antigen could be used to protect against very different pathogens, belonging to quite distant families or orders, and we are not aware of any previous use of an antigen from a phylum organism to immunize against diseases caused by microrganisms from another phylum This is somewhat surprising in view of the existence of conserved, highly immunogenic proteins (for instance, the HSPs) in many different pathogens, or compounds such as the peptidoglycans or the lipopolisaccharydes so widely shared among bacteria. In a way, an immunological dogma asks that vaccine specificity may be acquired only by the use of highly specific antigens, in a sort of opposing counterpart to natural immunity where non-specific, widely cross-reactive recognition is the rule – Lipolysaccharides and various glycans are indeed major stimulators of natural immunity (the so-called pathogen-associated microbial patterns, PAMP) through their binding to the family of Toll-like receptors TLRs and other receptors, such as, for instance, the Dectin-1 for fungal glucan (Brown and Gordon, 2005).

In a series of past and recent investigations aimed to find novel approaches to passive and active vaccination against human opportunistic fungi, we realized that

the above dogmatic specificity concept, contrasting natural with adaptive immunity, could be reversed by using a compound from another phylum organism to immunize against fungi. Thus, we proposed a beta-glucan constituent (laminarin) from the alga *Laminaria digitata* as a candidate antigen for a single vaccine potentially protecting against various, different fungal infections (Torosantucci et al. 2005). Importantly, our investigations also revealed that the algal, glucan-based vaccine elicited antibodies with a direct inhibitory activity against these pathogens, thus adding a critical advantageous requisite for vaccination of immunocompromised subjects. A convergent approach in the same, if not wider purpose, has been the use of a monoclonal antibody neutralizing a wide-spectrum antimicrobial killer toxin as immunizing antigen to raise anti-idiotypic antibodies mimicking the activity of the killer toxin on fungi (Polonelli et al. 1997). The two approaches might have shared a common component if it will be definitely proven that the anti-idiotypic, killer toxin-mimicking antibodies raised by the immunization with the killer toxin neutralizing monoclonal antibody recognize ß-glucan constituent as cognate antigen.

5.1. Beta-Glucan Constituents of Fungal Cell Wall

Beta-glucans are structurally complex glucose homopolymers, found in the cell wall of fungi, algae and bacteria (Stone 1992, Masuoka 2004). Their basic molecular structure is relatively homogeneous, although type of bonding, molecular mass and overall molecular configuration may be variable depending on the different microbial source (Bohn 1995). Biologically, they are well-known for their immunomodulatory and anti-tumor properties (Cassone 1987; Brown 2003; Masuoka 2004) but, to our knowledge, have never been considered as vaccine antigens but rather as immunomodulators or, more recently, as PAMP.

In the opportunistic fungal pathogen *Candida albicans*, β-glucans are major structural components, accounting for approximately 50–60% of cell wall dry weight. Based on different solubility in alkali and acid, Candida β-glucan has been differentiated into an alkali-soluble polymer of a relatively low molecular weight and a branched, acid-soluble molecule, both predominantly composed of β-$(1 \rightarrow 6)$-linked residues, and into an alkali-acid insoluble, highly branched complex, containing grossly equivalent amounts of β-$(1 \rightarrow 6)$ and β-$(1 \rightarrow 3)$ linkages in a complex with chitin providing form and structural integrity to the fungal wall (Chattaway et al. 1968; Cassone 1991).

It is generally accepted that in this fungus glucans preferentially enriched with β-$(1 \rightarrow 6)$- or β$(1 \rightarrow 3)$ linkages (possibly a family of distinct molecules, widely interconnected to each other) are differentially located and play distinct structural roles in cell wall architecture. Recent models of cell wall structure suggest that β-$(1 \rightarrow 3)$-linked glucan molecules form a three-dimentional matrix surrounding the fungal cell. At the inside, close to the plasma membrane, this skeletal framework is strengthened by chitin chains, whereas, at its outer edge, β-$(1 \rightarrow 6)$ glucan moieties link GPI-anchored cell wall mannoproteins to the skeletal framework (Klis 2001) (A scheme of cell wall organization in *C.albicans* is shown in Figure 1,
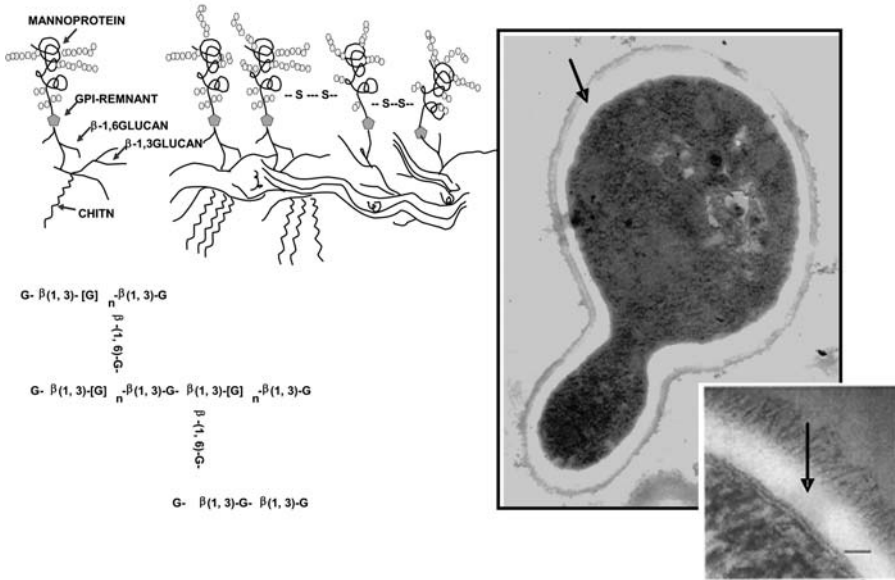
Cell wall structure in *C.albicans*

Figure 1. A shematic representation of the cell wall composition and structure in *C.albicans*. The arrows in the inset electron micrographs point to the inner layer of the cell wall where most of the glucan is present, some of it bound to chitin.(see however. Legend to Figure 2). The outermost fibrillar layer is considered to contain mostly mannoproteins. For references, see text

whereas Figure 2 shows the immuno-cytochemical detection of beta-glucan by the use of a specific murine monoclonal obtained by immunization with the Lam-CRM conjugate, see below).

While human pathogenic fungi contain both beta1–6 and beta 1–3 glucan, the expression and predominance of each of the two isomers is quite variable, depending on the fungus and its form of growth. In addition, beta-glucan can be replaced by alfa-glucan in some dimorphic fungi.

5.2.   Protection against Candida and Aspergillus Conferred by a Glucan Vaccine

Glucans are per se very poor saccharide antigens, probably the "dullest" of all, as being constituted by a homopolymeric sequence of α-glucopyranosyl residues (Fig. 1). Very low, exclusively of IgM isotype, antibody levels in mice are achieved by even the most aggressive immunization schedules with pure, soluble or particulate glucans as antigens (Bromuro et al. 2000). However, as other polysaccharides, glucans may become strongly immunogenic when conjugated with a protein carrier. The findings of low-level,anti-β-glucan antibodies, in the serum of normal healthy human subjects probably is a consequence of natural exposure to glucan-protein

Figure 2. Immunogold labelling of cell wall glucan in *Canida albicans* through the use of mAb1E12 which detects, with different affinity, both ß1–3 and 1–6 glucan configuration. Note that glucan miolecules are also present on cell wall surface. For further details, see the text and Ref. Torosantucci et al. 2005

complexes as those found in the cell wall of many fungi (Chaffin et al. 1998). Previous evidence indicated to us that a vaccine composed by intact Candida or Saccharomyces cells treated to expose glucan rather mannan on cell surface conferred a substantial degree of protection, and that anti-ß-glucan antibodies could have been involved in the protection (Bromuro et al. 2000) For all said above, we considered that a vaccine based on a glycoconjugate between a ß-glucan molecule and a carrier protein could allow simultaneous immunization and protection against a variety of pathogenic fungi. To test the strength of this cross-immunization or even transphyletic vaccination we elected to use an algal, laminarin, rather than glucan extracted from Candida or other fungi, also to avoid possible contamination with other immunodominant fungal antigens, e.g. mannoproteins. As a carrier protein, CRM197, a genetically-detoxified diphtheria toxoid, already safely used in other current vaccines was selected. Shortly, this novel glycoconjugate met all expectations in terms of immunogenicity and protection from both mucosal and systemic candidiasis in rodents, as well as systemic aspergillosis in mice. The protection was clearly due to anti-ß-glucan antibodies which bound preferentially to the growing hyphal cell wall, both in *C.albicans* and in *Aspergillus fumigatus*. Moreover, a IgG2b monoclonal antibody, raised in vaccinated mice and recognizing at high affinity ß1–3 glucan configuration, mimicked the protective effect of the immune sera, and also bound to the hyphal cells.(Torosantucci et al., 2005). Figures 3 and 4 exemplify
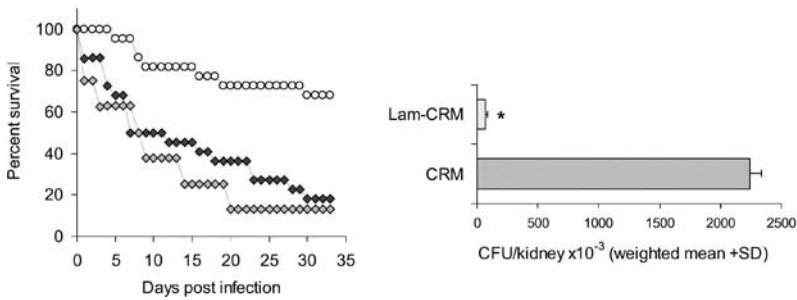
Figure 3. Protection induced by vaccination with the Lam-CRM conjugate as shown by Kaplan-Meyer survival curves (left panel) and Candida CFU counts in the mouse kidney (right panel). In the left panel,. the grey and blue dots refer to mice injected adjuvant or CRM protein, respectively, while in the right panel the grey color represent CFU of CRM-alone injected animals. In both panels, the yellow colours represent the values associated with Lam-CRM conjugate immuniozation. For further details, see Torosantucci et al. 2005

some of the immunogenic and protective activities induced by the vaccine. In particular, Figures 2 and 4 show that, in contrast with the common belief, ß-glucan molecules are present also on the cell surface of both Candida and Aspergillus, at least on the hyphal cells of these fungi, an observation in part matching the recent report of Dectin-1, a major glucan receptor, binding to cell surface of *C.albicans* (Gartner et al. 2005).

5.3.   What anti- β-Glucan Antibodies do for Protection

β-glucan constituents are therefore present on fungal cell surfaces, particularly on growing hyphae, thus they are accessible to antibodies, which can opsonize the cells and facilitate complement deposition, a process whose importance for protection is quite obvious and has been highlighted in several studies (Casadevall et al. 2004). Antibodies to cell surface components of *C.albicans* have been shown to favour both intracellular and extracellular killing of the fungus and, quite recently anti-ß_-glucan antibodies have been shown to increase the candidacidal activity of macrophages in vitro. Antibodies binding the hyphal form of growth could also perturb adherence and tissue invasion, as has been demonstrated recently with a mAb directed against a stress-mannoprotein of *C.albicans* (Moragues et al. 2003).

While all above may be contributory factors, we believe that other properties of these antibodies may be truly relevant for the fungal cross-species protection conferred by the vaccine.

5.4.   Hyphal Growth-Inhibitory Antibodies

In addition to the mechanisms already discussed, our data suggest that an additional mechanism for protection by anti- ß-glucan antibodies could operate in vivo. In
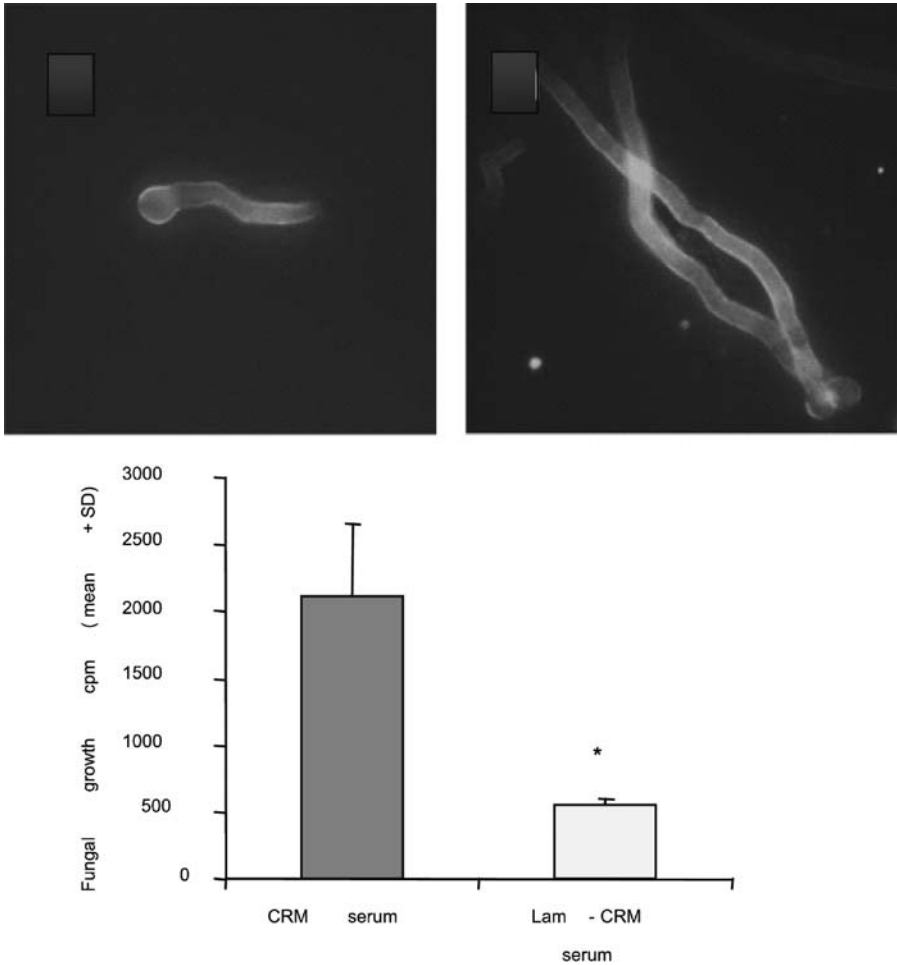
Figure 4. Binding (top panels) and in vitro hyphal growth inhibition (bottom panel) caused in *A.fumigatus* by incubation with immuneserum of mice vaccinated with the Lam-CRM conjugate. For further details, see Torosantucci et al. 2005

fact, the immune serum from vaccinated mice exerted a marked inhibition of Candida (and Aspergillus) hyphal growth in vitro, an effect that was also exerted by the affinity-purified IgG-rich fraction and that is in keeping with the preferential antibody binding to the hyphae. Together with previous data obtained with other yeast killer toxin–mimicking, anti-idiotypic antibodies our findings suggest that certain anti- ß-glucan antibodies may be endowed with direct inhibitory activity on fungi through some sort of interaction with such viability-critical molecules, which possibly include the oligosaccharide of nascent chains bound to the transg-lycosidases and glucan synthases.In accord with this hypothesis, recent data in our

laboratory have shown that the immune serum from Lam-Crm-vaccinated mice is able to inhibit certain stages of cell wall regeneration from protoplasts of *C.albicans*, coincident with beta-glucan deposition (unpublished data). The observation that these antibodies inhibit hyphal growth, possibly through inhibition of one critical component of cell wall machinery is of particular interest since, at least in *Candida albicans*, but probably also in Aspergillus (Stevens, 2004) hyphae appear to carry the main virulence traits, such as the adhesins and proteases, which contribute to fungus pathogenicity. In this context, these antibodies would work as a sort of cell wall inhibitory "antibiotics". In addition, the hyphae have been repeatedly shown to confer immunoevasion properties to the fungus, probably by activating the TLR2-mediated signaling pattern contrasting, through IL-10 production, the Th1 protective cytokine axis (see above). ß-glucan has been reported to activate this TLR2-dependent pattern through binding to dectin-1 receptor and, interestingly, in terms of immunoevasion, hyphae do not bind dectin-1 receptor (Gartner et al. 2005). Thus, in theory, anti-β-glucan antibodies may also exert protection by neutralizing the above signalling mechanism and shifting the cytokine profile toward the protective Th1 pattern, an observation already made, with other antibodies, in experimental cryptococcosis (Casadevall et al. 2004). Overall, anti-ß-glucan antibodies could be particularly protective by the expression altogether of their typical properties of immunomodulatory pro-defence components associated with some peculiar inhibitory properties featuring a sort of antibiotic action. Whatever the mechanism, this novel approach to a cross-immunizing vaccine may open the way to vaccination against at least some of the major opportunistic fungal agents of highly prevalent and incident diseases. This mostly whether it could be shown in future studies that the antibodies raised by this vaccine are still present in sufficient titer during immunosuppressive therapy and do not cause unbalance in the microbial flora and other untoward effects owing to their wide specificity. Our approach also provides for a novel vaccine which could be used to raise human or humanized antibodies for passive immunization, an approach which is now ongoing in our laboratories, and the outcome of which necessarily requires an epitope dissection and the precise identification of the cognate antigens within the beta1–3 and beta1.6 glucan molecules.

6.   CONCLUDING REMARKS

Previously neglected vaccines such as the antifungal ones are gaining steps in the public health priority scale. The increased awareness of the medical threat represented by fungal infections, the advances in the knowledge of how fungi cause disease and which immune response may keep them at bay, together with improved biotechnological approaches to candidate vaccine antigens and engineered antibodies have offered critical new tools to anti-fungal vaccine generation. Fungal vaccines may also benefit of the clearly increased advocacy by the public and private sectors of the theory and practice of vaccination with its unrivalled risk- and cost-benefit ratios. All this has brought into the field increased enthusiasm and

commitment. The discovery that antibodies may play a critical role for protection is also giving strong impetus to the field of passive vaccination, which may eventually prove to be the first vaccine application against fungal infections, also helped by the spectacular advances in the generation of human and humanized monoclonal antibodies and various technological fragments of them. Research on active and passive vaccination against fungi is also offering novel ideas and some innovative approaches to the other fields of vaccine research (Casadevall and Pirofski, 2006). Clearly, there is more hope now than few years ago about the chances of generating and getting approved by the regulatory authorities one or more antifungal vaccines, be active or passive, for use in humans in the next few years.

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