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Towards a carbohydrate-based vaccine against leishmaniasis

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Leishmania are digenetic protozoa, alternating between the promastigote, a free-living flagellate in the gut of the vector sandfly, and the amastigote, the obligatory intracellular form which resides in phagolysosomes of mammalian macrophages. Infection with this organism begins with the recognition of the host macrophage by the promastigotes, attachment and uptake by 'facilitated phagocytosis' [1]. The basic lesion in leishmaniasis is the infected macrophage. The infected macrophage displays parasite antigens on its surface (reviewed in [9]) and there is no reason why these should not be recognized by T cells in an MHC restricted manner.

Leishmania major infection of mice produces a range of disease patterns, similar to the situation in man, depending on the strain of inbred mouse. However, hypothyroid nude mice of both resistant and susceptible genotypes are highly susceptible, suggesting a role for T cell-dependent immunity in resistance to disease [3, 11].

In response to *L. major* infection, both delayed-type hypersensitivity (DTH) and antibodies are produced (reviewed in [11]). However, there appears to be no strict correlation between healing and development of cell-mediated immune responses, and progressive lesions may develop in the presence of DTH to crude antigen mixtures [11]. Animals of resistant genotypes recover from infection and are resistant to reinfection [9, 11]. Protection can be transferred with L3T4⁺, Ly2⁻ T cells to syngeneic recipients [11].

Vaccination against cutaneous leishmaniasis is feasible with crude antigens [reviewed in ref. 11]. The ideal vaccine would be a molecularly defined set of antigens, either involved in the induction of host-protective immune responses or critical to parasite uptake into the macrophage. We have focused on the characterization of the parasite molecules involved in recognition and uptake into host macrophages as potential vaccine candidates. Using the monoclonal antibody WIC-79.3, we have recently described an externally oriented, amphipathic membrane antigen of *L. major* which is shed into culture medium [5, 6]. This antigen had been shown previously to be part of a polymorphic family of carbohydrate antigens present in all *Leishmania*. Each antigen was shown to be species-specific and this has formed the basis for a serotyping system for the classification and diagnosis of leishmaniasis [17].

While the structure of this molecule in *L. major* is still unknown, and an area of considerable interest, it appears from our data that the molecule is anchored into the parasite membrane by covalently attached fatty acid. It contains sulphated and phosphated sugars, with galactose in a terminal position, accessible for recognition by ricin lectin and radiolabelled by [³H] sodium borohydride following galactose oxidase treatment [6]. A similar molecule has been identified in *L. donovani* and partially characterized by Turco *et al.* [18]. Preliminary data indicate that it may be similar in structure to bacterial lipopolysaccharides (S. J. Turco, personal communication).

Our studies examining the biological function of this glycolipid, or lipopolysaccharide molecule, indicate that it binds specifically to macrophages *in vitro* [6]. In addition, antibodies to the carbohydrate moiety block attachment of promastigotes to macrophages. Taken together, our data suggest that the *L. major* glycolipid is involved in macrophage recognition and attachment, and that this interaction occurs *via* the carbohydrate part of the molecule. Interestingly, the presence of this molecule, or parts of it, on the surface of *infected macrophages* can be inferred from immunofluorescence studies using monoclonal antibodies [2, 4]. Consequently, this antigen is biologically important as a recognition molecule, and immunologically important because of its expression on the surface of the infected macrophage, available for T-cell recognition.

Mice immunized with the glycolipid antigen purified from detergent lysates of *L. major* promastigotes using the monoclonal antibody WIC-79.3 (i.e. L-LPS) are resistant to subsequent infection with *L. major* [7, 8]. When injected with L-LPS plus the adjuvant, killed *Corynebacterium parvum*, intraperitoneally, mice of healer phenotype (e.g. C3H/He, C57BL/6) may be totally resistant in terms of lesion development. Doses used have been in the range of 2–10 µg L-LPS and 100–200 µg *C. parvum*. Immunized mice of non-healer phenotype (BALB/c and BALB/c H-2 congenic mice) generally show a delayed appearance of lesions and/or lesions that remain small in size. As

with crude antigen mixtures (including attenuated whole organisms), with or without adjuvants [7, 10, 12, 13], subcutaneous injections fail to induce resistance to disease in mice. The nature of this peculiar constraint imposed by route of injection remains unknown; it is also unknown whether it pertains to man.

No protective effect in mice has yet been demonstrated with the carbohydrate portion (CHO) of L-LPS prepared from promastigote culture supernatants and injected by any route, with any adjuvant or in any amount. In fact, CHO injected with Freund's complete adjuvant (FCA) will increase the duration of subsequent disease in C3H/He and C57BL/6 mice [8, 15] challenged with *L. major* promastigotes. Moreover, it can be shown readily by adoptive transfers in nude mice that BALB/c mice injected with CHO plus FCA have an increased frequency of disease-promoting ('suppressor') cells in their lymphoid organs [15]. Using *in vivo* titrations in BALB/c nu/nu mice, the frequency of disease-promoting cells (that from all other evidence in the cutaneous leishmaniasis-mouse system are likely to be Ly2⁻ T cells) is increased by about 10. Other unpublished and preliminary data suggest that injections of FCA plus the CHO purified from *L. donovani* culture supernatants, by affinity chromatography on monoclonal antibody WIC-108.3 increase the susceptibility of C3H/He mice to *L. major*. Thus there may be a sharing of 'disease-promoting epitopes' between the carbohydrate moiety of the L-LPS of these two leishmania species.

We have proposed [14, 16] that L3T4⁺ Ly2⁻ T-cell recognition of L-LPS, oriented in the membrane of infected macrophages through its lipid component, results in the release of mediators of macrophage activation (such as INF- γ and others) with subsequent parasitostatic, if not parasitocidal, effects. In contrast, the CHO component of L-LPS should be oriented quite differently following binding to its receptor on the surface of macrophages. Both recognition of L-LPS and its CHO component should be class II MHC (Ia) restricted. Clearly, like L-LPS, any antigen recognized by appropriate T cells and present in appropriate amounts on the infected macrophage surface should serve as a target of aggressive T cell-mediated attack. If T-cell receptors stabilize associations between antigen and Ia molecules at the cell surface, then *amounts* of lipophilic antigen at the infected macrophage surface (in the absence of any real affinity of most antigens for Ia) will be the critical factor.

The nature of disease-promoting immunity remains unknown. Antibodies appear to play no role in either resistance or disease-promoting immunity. An hypothesis we favour at the moment is that CHO recognition by IFN- γ producing T cells at the surface of *uninfected* macrophages in lesions increases the expression of the macrophage receptors to which CHO binds in the process of 'facilitated phagocytosis' of leishmania. This increased display of receptors enables parasites released from destroyed macrophages to gain

entry quickly and thereby facilitates spread of infection. Clearly, little more can be gained from speculation in the absence of (a) structural data on the L-LPS and its carbohydrate fragment(s) released by phospholipase treatment, (b) T-cell clones with specificity for these antigens and (c) clonal analyses of T-cell populations in immunized mice of various genotypes using defined antigens.

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REFERENCES

1. Chang, K. P., and Fong, D. Cell biology of host-parasite membrane interactions in leishmaniasis. In *Cytopathology of Parasitic Disease*, Pitman Books, London, Ciba Foundation Symposium 99, (ed. D. Evered and G. M. Collins) 1983, pp. 113-37.
2. DeIbarra, A., Howard, J. G., and Snary, D. Monoclonal antibodies to *Leishmania tropica major*: specificities and antigen location. *Parasitol.*, **85**, 523-31 (1982).
3. Handman, E., Ceredig, R., and Mitchell, G. F. (1979). Murine cutaneous leishmaniasis: disease patterns in intact and nude mice of various genotypes and examination of differences between normal and infected macrophages. *Aust. J. Exp. Biol. Med. Sci.*, **57**, 9-29 (1979).
4. Handman, E., and Hocking, R. E. Stage-specific strain-specific, and cross-reactive antigens of *Leishmania* species identified by monoclonal antibodies. *Infect. Immun.*, **37**, 28-33 (1982).
5. Handman, E., Greenblatt, C. L., and Goding, J. W. An amphipathic sulphate glycoconjugate of *Leishmania*: characterization with monoclonal antibodies. *EMBO J.*, **3**, 2301-6 (1984).
6. Handman, E., and Goding, J. W. The *Leishmania* receptor for macrophages is a lipid-containing glycoconjugate. *EMBO J.*, **4**, 329-36 (1985).
7. Handman, E., and Mitchell, G. F. Immunization with *Leishmania* receptor for macrophages protects mice against cutaneous leishmaniasis. *Proc. Natl. Acad. Sci. USA.*, **82**, 5910-14 (1985).
8. Handman, E., and Mitchell, G. F. *Leishmania*-macrophage interaction: role of parasite molecules in infection and host protection. In *Molecular Strategies of Parasitic Invasion*, UCLA Symposia on Molecular and Cellular Biology, New Series, Vol. 42 (ed. N. Agabian, H. Goodman and N. Noguiera), Alan R. Liss, New York, 1987 (in press).
9. Handman, E. Leishmaniasis: antigens and host-parasite interactions. In *Parasite Antigens: Toward New Strategies for Vaccines*. (ed. T. W. Pearson), Marcel Dekker, New York and Basle, 1986, pp. 5-48.
10. Howard, J. G., Liew, F. Y., Hale, C., and Nicklin, S. Prophylactic immunization

- against experimental leishmaniasis. II. Further characterization of the protective immunity against fatal *Leishmania tropica* infection induced by irradiated promastigotes. *J. Immunol.*, **132**, 450–5 (1984).
11. Howard, J. G. Immunological regulation and control of experimental leishmaniasis. *Int. Rev. Exp. Pathol.*, **28**, 80–113 (1986).
 12. Mitchell, G. F., Handman, E., and Spithill, T. W. Vaccination against cutaneous leishmaniasis in mice using nonpathogenic cloned promastigotes of *Leishmania major* and importance of route of injection. *Aust. J. exp. Biol. Med. Sci.*, **62**, 145–53 (1984).
 13. Mitchell, G. F., Handman, E., and Spithill, T. W. Examination of variables in the vaccination of mice against cutaneous leishmaniasis using living avirulent cloned lines and killed promastigotes of *Leishmania major*. *Int. J. Parasitol.*, **15**, 677–84 (1985).
 14. Mitchell, G. F., and Handman, E. T-lymphocytes recognize *Leishmania* glycoconjugates. *Parasitology Today*, **1**, 61–3 (1985).
 15. Mitchell, G. F., and Handman, E. The glycoconjugate derived from a *Leishmania major* receptor for macrophages is a suppressogenic, disease-promoting antigen in murine cutaneous leishmaniasis. *Parasite Immunol.*, **8**, 255–63 (1986).
 16. Mitchell, G. F. Cellular and molecular aspects of host–parasite relationships. In Proceedings of the VI International Congress of Immunology, *Progress in Immunology* (VI) (ed. B. Cinader and R. G. Miller), Academic Press, Florida, 1987 (in press).
 17. Schnur, L. F. The immunological identification and characterization of leishmanial stocks and strains, with special reference to excreted factor serotyping. In *Biochemical Characterization of Leishmania* (ed. M. L. Chance and B. C. Walton), UNDP/World Bank/WHO, Geneva, Switzerland, 1982, pp. 25–48.
 18. Turco, S. J., Wilkerson, M. A., and Clawson, D. R. Expression of an unusual acidic glycoconjugate in *Leishmania donovani*. *J. Biol. Chem.*, **259**, 3883–9 (1984).

DISCUSSION – Chaired by Dr J. B. Robbins

HANDMAN: Our problems began, though, when we tried to induce the same type of protection using the water-soluble carbohydrate antigen alone. In this example we used genetically resistant mice which develop a lesion that will eventually heal so the uninjected mice or the ones injected with the adjuvant alone eventually heal. However, mice that were immunized or vaccinated with the carbohydrate in Freund's complete adjuvant, were not only not protected from disease but, in fact, the disease was exacerbated. I could put forward several hypotheses for what causes this peculiar type of immune response but I won't because we have no evidence for any of them and this is where I would like to get some feedback from you people who have worked with carbohydrate antigens for a long time. I think that it may be a matter of antigen presentation – that the glycolipid antigen is anchored in the membrane of the antigen-presenting cell in such a way that it can interact with the host-histocompatibility antigen in a way that will then induce a T-cell protective immune response, whereas the carbohydrate alone may

interact with the initial ligand, the molecule it normally binds to, but then it probably doesn't interact with class II antigen in the same way that the glycolipid does. All of this though is speculation and we really don't understand it. What we do understand is that we will not rush into human vaccination trials until we know how to make a very stable preparation to ensure that we will induce the appropriate immune response.

ADA: If you immunize your mice with the soluble carbohydrates and then immunize with the lipid, what happens?

HANDMAN: We are doing these experiments now in both directions to see if the carbohydrate overrides protection or if the glycolipid overrides suppression.

ADA: One possibility might be that you are causing tolerance to suppressor-T cells, you are activating suppressor-T cells by giving the free carbohydrate. Now, if that were the case, if you gave the free carbohydrate, then treated with cyclophosphamide to get rid of those suppressor-T cells then your mice might become more responsive to the glycolipid. I would think that if you did those two sets of experiments you might get an indication whether that was what was happening.

HANDMAN: Well, Graham Mitchell is doing this experiment now and we should know soon.

HOOKE: Have you thought about incorporating the glycolipid into liposomes and looking at the immune response to that?

HANDMAN: Yes. In fact my very first experiment was to use a lipid adjuvant called Lipovant, sold by Accurate Biochemicals in the USA, which is egg lecithin and, in fact, I found that the adjuvant alone, or the liposomes alone, exacerbated the disease and we have not used them since, but we will try and prepare our own liposomes.

KABAT: I would like to say, I will say more about it this afternoon, that Charles Wood and Eric Lai in my laboratory have been quite successful in getting antibodies to the isomaltose oligosaccharides coupled to stearylamine, so that you could take your recovered carbohydrate, couple it to something like stearylamine and make another type of glycolipid which we found to be a very good antigen, which seems to be thymus independent.

MORENO: Just for clarification, did you follow the antibody titres in those animals immunized with soluble polysaccharide in complete Freund's adjuvant?

HANDMAN: Yes. I must stress though, that the antibodies are not believed to play a role in protection in cutaneous leishmaniasis, it is a T-cell mediated type of protection, but I did follow the antibody titres and the mice made no antibodies whatsoever, whereas they did make antibodies when immunized with the glycolipid.

MÄKELÄ, Helena: In one of your slides you had in parenthesis an indication that this glycolipid antigen would also be active as a virulence factor within

the macrophages. I wonder whether you have any evidence of the mechanism of this activity?

HANDMAN: We have been trying to produce parasite mutants that lack this molecule and we found one such parasite which is taken up by macrophages, probably by a different mechanism, and is killed inside macrophages very rapidly. There are two possibilities and I can't distinguish between them. One is that a parasite which has this glycolipid molecule on its surface will interact with a set of receptors on macrophages which will target it into a compartment in the macrophage where it can survive. The parasite that does not have this antigen on the surface will interact with a different set of ligands on the macrophage and will end up in a different compartment and be killed, let us say by lysosomal enzymes for example. The other possibility is that parasites with or without this coat will end up in the same compartment, but this glycolipid protects them from killing by lysosomal enzymes. I cannot, at the moment, distinguish between them, but when I took these mutant parasites and incorporated into their membrane the purified lipids from the wild-type parasite, they survived much better in macrophages. So I think that this molecule is also involved in survival, I am still not sure exactly at what level.

MÄKELÄ, Helena: We have made rather similar observations, still at a preliminary stage, with *Salmonella* bacteria, a bacterial parasite which, however, lives in the same compartment in the macrophages as does the *Leishmania*, and obeys very much the same rules. We have looked at mutants that are devoid of a glycolipid antigen called the enterobacterial common antigen; to our great surprise they were very much more sensitive to the intracellular compartment than the normal ones and we are at the moment in the process of finding out what the mechanism of that is.

HANDMAN: It would be intriguing if they were targeted to a different compartment. There was a time when we used to believe that a lysosome is a lysosome is a lysosome, now I think there is more and more evidence that there are various acidic compartments and they are not all the same.

SÖDERSTRÖM: Did you look at the presence of these receptors also in the Langerhans cells, in the skin?

HANDMAN: No, we have not yet.

SUTHERLAND: You mentioned a disaccharide repeating unit. Can you give us any indication of either the composition or the structure of this unit?

HANDMAN: Well these are not my own data. It is a preliminary personal communication from Sam Turco so I would like to be very careful. He thinks that, in the case of *Leishmania donovani* – the parasite causing visceral disease in man, it is a (mannose (β -1.4) galactose) disaccharide unit which is phosphodiester-linked. But I can't say any more.

ROTTA: I might have missed this, but I am wondering whether you looked in your immunized animals for cell-mediated immunity reactions?

HANDMAN: No, we have not looked at the mechanism which induces host

protection in mice vaccinated with the lipid. We know that T cells from these mice respond to the glycolipid *in vitro*. We are now in the process of making T-cell lines from these mice, but we haven't actually done any *in vivo* experiments yet.

People who recover from cutaneous leishmaniasis are protected against future infections, and based on this some traditional societies have for centuries intentionally infected vulnerable people in inconspicuous locations; bedouins for example have sandflies bite their children's buttocks, and people in the middle east have transferred fluid from lesions on infected people to non-infected people using thorns.[52][53] This.Â Preclinical work with genetically-modified live attenuated parasite vaccines was conducted in the 1990s and 2000s, as did work with synthetic peptides, recombinant proteins, glycoproteins and glycolipids from leishmania species, and naked DNA.[52][54] As of