Analysis of idiotypes expressed by anti-mycobacterial mouse monoclonal antibodies using rabbit antisera

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Abstract

Rabbit antisera against twelve mouse monoclonal antibodies to mycobacterial antigens showed idiotypic-specificity following cross-absorption with normal mouse globulin. The functional aspects have been partly evaluated so far with two of these reagents. The representation of ML04 idiotypic appeared to be associated with Igh alleles. Injection of mice with Rb04 anti-Id in incomplete Freund's adjuvant induced Id⁺ serum levels but without corresponding antigen binding specificity. In contrast, Rb71 anti-Id revealed internal image properties corresponding to the homologous 38kd protein antigen of tubercle bacilli. This was demonstrated by the potency of Rb71 to sensitize mice *in vivo* and to elicit T cell proliferative and DTH responses.

Introduction

It is generally believed that leprosy and tuberculosis occur only in a minority of infected subjects. Therefore, the development of disease may possibly be due to a defect in the host immune system. While the mechanisms are not fully understood, it is likely that the defect is antigen specific since most patients are not generally immunocompromised. Interest has focused on the T cell-macrophage inter-relationship since these cells were shown to be responsible for protection in mouse models of infection with tubercle bacilli and *M. leprae* (1,2).

Antibody levels are increased in lepromatous leprosy (3-5), as well as in pulmonary tuberculosis (6,7), but except a few studies (8,9), they have so far not been considered to play a role in the pathogenesis of these diseases. We initiated the study of idiotypes expressed on anti-mycobacterial antibodies with the view to bridge over this gap in our knowledge. Our approach seemed justified by experimental studies which demonstrated that various manipulations of the B cell immune system can profoundly perturb T-cell mediated immune reactions (10-12). In this context, it is also intriguing that the extent of chronic granulomatous reactions to BCG in mice (with associated T cell anergy) has been linked with the Igh locus which controls the structure of immunoglobulin (Ig) heavy chains (13).

It was reported in previous studies that polyclonal antisera raised against Ig idiotypes, can induce *in vivo* DTH reactions, or T-helper mediated responses with specificity relating to the original antigen (14-18). The broad aims of this study were therefore to explore, in similar experimental models, the biological potency of anti-idiotypic reagents directed towards the monoclonal antibodies (MABs) to mycobacterial antigens. This paper describes the pre-

liminary results from experiments which will be fully reported elsewhere (Praputpittaya and Ivanyi, in preparation).

Production of rabbit anti-idiotypic antisera

Globulin fractions precipitated by 16 % Na_2SO_4 from the ascitic fluid of twelve hybridoma MABs (19-23) were injected to New Zealand White rabbits in incomplete Freund's adjuvant (IFA). Antisera were extensively absorbed by affinity chromatography on normal mouse Ig (NMIg) coupled CNBr-Sepharose 4B columns. The idiotype restricted specificity of absorbed sera was established by their binding to polyvinyl 96 well plates coated with the homologous MAB-Ig, and the lack of binding to NMIg or heterologous MAB-Ig coated plates. Furthermore, the anti-Id sera were tested by two immunoassays schematically described in Table 1.

Table 1. Competition immunoassays employed for the characterisation of anti-Id antibodies.

Stage of the test	Assay	
	Paratope (SACT)	Idiotypic (AICT)
Coating of polyvinyl 96 well plates:	Mycobacterial soluble extract (MLSE, MTSE)	Rabbit anti-Id serum or Ig
Serial dilution of:	test sera (antibodies)	
^{125}I -labelled reagent	Id ⁺ -MAB-Ig (Na_2SO_4 ppt)	

SACT = serum antibody competition test

AICT = anti-idiotypic competition test

MLSE = *Mycobacterium leprae* soluble extract

MTSE = *Mycobacterium tuberculosis* soluble extract

Antibody levels are expressed as reciprocal serum dilutions (titres) giving 50 % inhibition of binding (ID_{50}).

The inhibitory activity of anti-Ids in the SACT assay localised the Ids within the antibody combining site whereas the AICT assay determined the binding specificity when comparing pairs of MAB-anti-Ids. The basic characteristics of tested MABs are summarised in Table 2. All anti-Ids blocked the MAB-antigen binding and their potency is expressed by titre in the SACT assay. The idiotypes of two groups of four MABs each of overlapping paratope specificity towards the 12kd and 35kd *M. leprae* specific epitopes respectively were analysed by the AICT and SACT assays. One Id shared between three MABs and another distinct Id were identified in both groups (Table 2). The structural localisation of Ids was examined by the immunoblot analysis of separated H&L chains from reduced/alkylated MAB-Ig following SDS-polyacrylamide gel electrophoresis. The results showed the binding of Rb06 to H-chains and Rb78 to L-chains, whereas all other anti-Ids reacted only to intact Ig molecules.

Expression of Ids in genetically defined mouse strains

In initial studies, the occurrence of Ids in the antibody repertoire of MLSE-immunized mice

Table 2 Review of anti-idiotypic antibodies

Code of rabbit anti-Id	MAB(Ig)	Antigen ³⁾	Specificity of MAB			Idiotypic expression	Anti-Id SACT titre
			M.lep.	M.tuber.	Others.		
Rb06	ML06 (G1)	12kd	+	—	—	H-chain	8 000
Rb10	ML10 (G2a) ¹⁾					Ig	5 000
Rb04	ML04 (G1) ²⁾	35kd	+	—	—	Ig	10 000
Rb38	ML38 (G1)					Ig	3 500
Rb30	-ML30 (G1)	65kd	+	+	broad	Ig	20 000
Rb34	ML34 (M)	25-40kd	+	+	broad	Ig	3 500
Rb02	ML02 (G3)					Ig	600
Rb23	TB23(G1)	19kd	—	+	limited	Ig	700
Rb78	TB78 (G1)	65kd	—	+	limited	L-chain	2 700
Rb68	TB68 (G1)	14kd	—	+	M.bovis	Ig	100
Rb71	TB71 (G2b)	38kd	—	+	—	Ig	700
Rb72	TB72 (G1)					Ig	500

1) overlapping idotype on ML05 (G2a) and ML12 (G2b)

2) overlapping idotype on ML03 (G1) and ML11 (G1)

3) All antigens are proteins except the 25-40kd lipoarabinomannan-B (24).

was determined. In view of the genetic control of immune responses to mycobacterial antigens by H-2 and non-H-2 genes (25-28), it was of interest to examine the genetic association of Ids in various inbred strains. Mice congenic for alleles controlled by Igh or H-2 loci were immunized with the soluble extract from *M.leprae* (MLSE) and serum anti-35kd antibodies were examined quantitatively (Table 3). The results indicated that ML04-paratope antibody titres followed a hierarchy which was linked with the Igh locus, but unrelated to the H-2 haplotype. The Rb 04-idiotypic also followed a similar pattern but quantitatively less pronounced. However, this genetic association was not apparent in the overall antibody binding potency tested with MLSE-coated plates.

Table 3 Genetic control of the 35kd (ML04) antibody response

Strain	H-2	Ig-h	Paratope ID ₅₀ ×10 ⁻³	Idiotypic % Binding 1/20 Serum dil.	MLSE % Binding 1/500 dil.
BALB.B	b	a	2.95	28.5	24.4
BALB.K	k	a	1.79	42.3	10.7
BALB.C	d	a	1.01	32.5	62.0
CBA.AK	k	d	1.28	32.9	13.7
CBA.Ca	k	j	.89	44.2	24.4
CBA.101	k	b	.51	17.7	7.6
B10.D2/N	d	b	.30	19.1	44.5
CB20	d	b	.26	12.4	50.0

It is of interest that patients with lepromatous leprosy have high levels of antibodies with specificities restricted only to some of the several antigenic constituents of *M. leprae* (29). Since the ML04 paratope is represented at high titre in these individuals (5), further study of genetic control of this specificity in mice may help to elucidate the mechanisms which determine its immunodominance.

The nature of Id+ («Ab3») response in mice injected with rabbit anti-Id serum

Previous studies had shown that injection of anti-Id antibodies into animals can induce the production of serologically detectable Id⁺ molecules (14,30-32). In these experiments, a certain proportion of Id+Ig was shown to bind with homologous antigen, whilst significant amounts of the Id can be expressed on antibodies of combining site specificity directed to other underdefined antigens. Whilst the mechanisms regulating the relative representation of Ids in a heterogeneous immune repertoire are poorly understood, they are of fundamental importance for the selection of those anti-Ids which carry the «internal image» and could serve as «surrogate» antigens.

The rabbit anti-Id serum Rb04 was injected (50μl) combined with IFA to BALB/c mice. Subsequently, mice were challenged with MLSE (50μg) initially with and later without IFA (Table 4). Sera harvested at various intervals were titrated in competition assays to determine the Id⁺ and paratope⁺ molecules. The results showed significant Id⁺ but no paratope activity in mice injected with Rb04/IFA alone. Paratope titres were demonstrable only in MLSE/IFA immunized mice but without a significant difference between the Rb04 and NR1g (control) primed groups. Moreover Rb04/IFA failed to sensitize for challenge with soluble MLSE.

Table 4. Induction of «Ab3» response by injection of BALB/c mice with Rb04 anti-Id.

Rb04-IFA	Injections (ip):		Serum ID ₅₀ titre	
	MLSE-IFA	MLSE-sol.	ML04-Id	ML04-Paratope
+	+	—	100	250
—	+	—	<10	80
+	+	+	200	1000
—	+	+	<10	400
+(2x)	—	—	100	<10
+(2x)	—	+	200	<10
—	—	—	<10	<10

The specificity of antibodies in the sera from double immunized mice was further examined by cross-absorption on either MLSE or Rb04 CNBr-Sepharose 4B column (Table 5). The results clearly showed the presence of two independent populations of molecules with either idiotype or paratope (antigen binding) specificities. Taken together, these experiments suggested that the pronounced Id⁺ response to Rb04/IFA was completely devoid of any binding specificity with the MLSE-derived 35kd antigen.

The following experiment was set up to investigate whether the Id⁺ response to Rb04/IFA could be regulated by mechanisms which operate in murine responses to protein antigens; namely, that the response to antigen-adjuvant injection can be abrogated by prior injection of soluble antigen (33,34). Soluble Rb04 (2x50μl) was given prior to challenge of mice with Rb04/IFA (Table 6). This protocol resulted in significant suppression of the Id⁺ serum values

Table 5. Dissociation between Rb04-Id⁺ and 35 kd antigen specific antibodies

Serum from Rb04 (anti-Id) plus MLSE(Ag) injected mice	ID ₅₀ serum titre	
	Paratope	Idiotope
Untreated	1070	270
Absorbed: MLSE CNBr-Sepharose 4B	<10	260
Absorbed: Rb04 CNBr-Sepharose 4B	895	<10

Table 6. Modulation of the «Ab3» response induced by Rb04 anti-Id.

Group	CY 50mg/kg -23d	Rb04 sol. -21,-14d	Rb04 /IFA od	Antibody level (+10d) + SE*) % Inhibition in AICT	% Binding to NRIg	
1	—	—	—	37.5±0.6	26.0±0.8	
2	—	—	+	62.8±2.3	53.1±2.8	} p< .001 } NS
3	—	+	+	31.5±2.1	52.1±1.7	
4	+	+	+	66.4±5.8	59.2±1.7	

*) at 1/20 serum dilution.

(group 3). In accordance with the response to protein antigens (35) the suppressive effect of Rb04 without IFA was abrogated by prior injection of cyclophosphamide (group 4, Table 6). Injection of Rb04/IFA also induced antibodies binding to normal rabbit Ig; however this response was not suppressed by prior injection of Rb04 alone. The CY-sensitive mechanism which implies the role of T suppressor cells (36) may play a regulatory role in response to the anti-Id-specific structure of the rabbit antibody.

«Internal image» activity of Rb71 anti-Id for T cell proliferative and DTH reactions

Experiments on the modulation of human T cell proliferative responses to the soluble extract from *M. tuberculosis* (MTSE) revealed that Rb71 anti-Id stimulated the *in vitro* ³H-TdR uptake by peripheral blood mononuclear cells from BCG-vaccinated donors (37). Stimulation by Rb71 anti-Id associated with responsiveness to the corresponding 38kd protein antigen. Furthermore, T cell clones were established which proliferated to either Rb71 or 38kd antigen only in the presence of irradiated autologous antigen presenting cells. Subsequent experiments in mice aimed to confirm and expand the analysis of the «surrogate» antigen characteristics of Rb71 anti-Id.

Mice were sensitized by subcutaneous injection of 50µg MTSE/IFA or Rb71/IFA and spleen cells were harvested for *in vitro* study using standard microwell culture techniques. Stimulation of ³H-TdR uptake by spleen cells from MTSE/IFA immunized mice was achieved by Rb71 but not by Rb72, Rb78, Rb23, Rb68, and Rb34. The proliferative response to Rb71 (affinity purified on a column of TB71-Ig coupled to CNBr-Sepharose) was abrogated by prior treatment of spleen cells with anti-Lyt 1.2 serum and complement (Table 7). These results suggest that the responding cells belong to the T helper/inducer subset. In a separate

experiment, spleen cells taken from Rb71/IFA injected mice showed a significant proliferative response to the purified 38kd protein. Thus, Rb71 anti-Id had imparted the 38kd antigen activity as the immunogen *in vivo* as well as the inducer of T cell proliferation *in vitro*.

Table 7. Stimulation of splenic T cells from MTSE-sensitized mice with affinity purified Rb71 anti-Id.

Exp	Priming	Cell Treatment plus C'	³ H-TdR, x 10 ⁻³ cpm ; 7 day culture				
			—	NR Ig	Rb71	38kd	MTSE
1	MTSE	—	0.93	0.86	21.53	NT	51.65
	MTSE	a-Lyt 1.2	1.59	0.72	2.46	NT	3.05
2	MTSE	—	3.66	0.86	10.96	15.75	11.10
	Rb71	—	3.55	13.20	15.43	18.32	5.66

Table 8. Antigen surrogate activity of Rb71 anti-Id for DTH responses of BALB/c mice

Sensitisation with soluble extract or rabbit Ig	Footpad swelling (mmx10 ⁻² ± SE) elicited by:		
	MTSE	Rb71	NRG
M.tuberculosis	77±6*	51±3*	17±2
M.bovis	93±6*(1)	72±5*	7±2
M.leprae	55±2*(1)	23±4	17±2
Rb71 anti Id	47±3*	NT	NT
NRG	18±8	NT	NT

* p<0.01 when compared with NRG (normal rabbit Ig).

(1) Elicitation with soluble extract homologous with the sensitising species of mycobacterium

Mice were sensitised for delayed type hypersensitivity (DTH) by subcutaneous injection of mycobacterial antigens (50µg) or anti-Id (5µg) emulsified in IFA, two days after pretreatment with 50 mg/kg cyclophosphamide (the latter is known to amplify mouse DTH reactions). The eliciting footpad injections of 10µg MTSE or 5µg rabbit Ig were given two weeks after priming and footpad swelling reactions were measured after 24 hours (Table 8). Rb71 elicited significant DTH reactions in mice sensitised with soluble extracts from *M.tuberculosis* or *M.bovis*, but not with *M.leprae*. Although MAB-TB71 reacts only weakly (10 % when compared with *M.tuberculosis*) with *M.bovis*, there is an apparently pronounced cross-reactivity in the recognition of the 38kd antigen by T cells. This result is in accord with the observed proliferative response of lymphocytes from BCG vaccinated human subjects to the 38kd protein (22). In the reverse combination, sensitisation with Rb71 resulted in a significant DTH response elicited by MTSE (containing the 38kd antigen) whilst normal rabbit Ig failed to sensitise.

Concluding comments

Results have been presented which demonstrate the contrasting behaviour of two anti-idiotypic antibodies. Rb04 effectively stimulated an Id⁺ serum Ig response, but without internal image of the corresponding 35kd protein antigen of *M. leprae*. On the other hand, antibody Rb71 could sensitize and elicit T-cell mediated proliferative and DTH reactions specific for the homologous 38kd protein antigen. The interpretation of these findings should be considered on the basis of mechanisms which determine the immunodominance of B cell or T cell stimulatory epitopes of protein antigens. It is generally believed that «hapten» and «carrier» like paired structures are mutually exclusive. This is expected to be the outcome of the distinct recognition mechanisms operated by B and T cells respectively. Consequently, idiotypic domains within the combining site of the majority of antibodies would not be shared with the T cell receptor. However, it is conceivable that in certain instances antibodies can be directed towards the same epitope which is presented in association with MHC Class II molecules on the surface of accessory cells to T cells. In this case the antibody combining site and the T cell receptor may contain a common idiootype which is complementary for the same epitope of the antigen. This hypothesis needs verification by further structural and functional analysis of the Rb71 idiootype. Moreover, the proposed interpretation when tested experimentally, may provide a rationale for a more predictable selection of such anti-idiotypic antibodies which carry the internal image of T cell epitopes.

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