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Study of idiotypes expressed by monoclonal antibodies to the 35 kD and 12 kD antigens of *Mycobacterium leprae*

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SUMMARY

Rabbit antisera were raised against four monoclonal antibodies (MoAb) binding with the 35 kD protein and four MoAb binding with the 12 kD protein antigen of *Mycobacterium leprae*. Antisera showed idiotype (Id) specificity following cross-absorption with normal mouse globulin. One Id on a single MoAb and another Id shared between three MoAb were identified for each group. Functional studies were carried out with the Rb04 anti [anti-35 kD] specificity. The expression of this Id and paratope in antigen immunized mice was associated with Igh alleles. Inoculation of mice with anti-Id Rb04 induced an 'Ab3' serum response of corresponding Id specificity only when the anti-Id was given in emulsion with incomplete Freund's adjuvant (IFA). Conversely, prior injection of soluble anti-Id inhibited the subsequent Ab3 response to Rb04/IFA. Moreover, the suppressive effect of soluble anti-Id was abrogated by prior injection of 50 mg/kg cyclophosphamide. These results indicate that regulatory mechanisms similar to those involved in antigenic stimulation may explain the stimulatory or suppressive potency of anti-Id antibodies. Finally, the Ab3 responses to the two tested anti-Ids did not contain any antigen binding activity.

Keywords idiotypes *Mycobacterium leprae* monoclonal antibodies

INTRODUCTION

Immunization of mice with killed *Mycobacterium leprae* can induce protection towards foot-pad challenge, but the antigens involved in the response have not been identified (Shepard, Walker & Van Landingham, 1978). Construction of an 'ideal' subunit vaccine would need to omit potential suppressor cell-inducing epitopes (Bloom, 1986; Modlin *et al.*, 1986; Ottenhoff *et al.*, 1986) and the inclusion of polypeptides containing merely the protective epitopes. However, synthetic peptide vaccine design has a drawback in that the technique can produce only linear antigenic determinants but not those involving distant residues brought together by natural folding of the native protein molecule. The alternative technology of gene cloning yielded a DNA expression library of *M. leprae* but at least one specific epitope carried by the 35 kD protein antigen has so far failed to express (Young *et al.*, 1985).

The concept of using anti-idiotype antibody, containing an internal image of antigen, for vaccine design carries the potential of overcoming the structural limitations of the other technologies and possibly even of the restrictions by host immune response genes (Roth *et al.*, 1985). Administration of anti-Id antibody *in vivo* can result in either a suppression or an enhancement of a

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corresponding Id, with or without any change in the magnitude of the immune response to the original antigen (Eichmann & Rajewsky 1975). The expectation that anti-Id antibodies could resemble antigenic epitopes has been fulfilled in animals which had not been exposed to the antigen before and particularly for protective immunity towards infection with *Streptococcus pneumoniae* (McNamara, Ward & Kohler, 1984), *Trypanosoma rhodesiense* (Sacks, Esser & Sher, 1982), hepatitis B virus (Kennedy *et al.*, 1986), reovirus (Sharpe *et al.*, 1984) and poliovirus (UytdeHaag & Osterhans, 1985). However, the appearance of an antigen internal image in anti-Id antibodies is yet not a predictable phenomenon and the relationship to the frequency of Id in the immune repertoire or the quality of anti-Id antibodies is poorly defined.

The aim of this study has been to define the nature and repertoire of idiotypes expressed on anti-*M. leprae* monoclonal antibodies, and to explore the biological activities of these anti-idiotype antibodies.

MATERIALS AND METHODS

Mycobacterium leprae *soluble extract (MLSE). Mycobacterium leprae* sonicates (batches 47, 51 and 52) were kindly provided by Dr R.J.W. Rees, WHO Bank, Mill Hill, London. This material was derived from *M. leprae* isolated from livers of infected armadillos irradiated with 2·5 mega-rads ^{60}Co, ultrasonicated and centrifuged at 100,000 *g* for 60 min. Supernatants were adjusted to 1 mg/ml protein concentration, and kept at −20°C.

Monoclonal antibodies. Monoclonal antibodies (MoAb) to antigens of *M. leprae,* were produced as a globulin fraction precipitated by 18% Na_2SO_4 from the ascitic fluids of mice injected with hybridoma cells (Ivanyi *et al.*, 1983; Ivanyi, Morris & Keen, 1985).

Production of rabbit anti-idiotype antisera. Rabbits of the New Zealand or Hartley White strain were immunized intramuscularly in both hind legs with 2·5 mg MoAb emulsified in incomplete Freund's adjuvant (IFA) (1 : 1 volume ratio). Booster injections of soluble globulin were given after 6 weeks and after 4 months, subcutaneously. Rabbits were bled 10 days later, antisera were separated from clotted blood and stored at −20°C.

Antisera were rendered specific for the idiotypic determinant of the immunizing MoAb by repeated absorption on a normal mouse globulin (NMG) coupled CNBr-Sepharose 4B (Pharmacia Sweden) column. Coupling of NMG to beads was carried out essentially by the technique recommended by the manufacturer. Cross-absorbed rabbit anti-Id sera were designated as Rb04 for anti-ML04, Rb10 for anti-ML10 etc.

Binding radioimmunoassay (BIA). Globulins were iodinated with Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycouril) essentially by the method of Fraker & Speck (1978). Flexible polyvinyl microtitre plates (Dynatech) were coated overnight at 4°C with 30 μg/ml of MLSE or 10 μg/ml normal rabbit globulin (NRbG) at 50 μl/well. Plates were washed three times with phosphate-buffered saline (PBS) and blocked by 3% bovine serum albumin in PBS (BSA/PBS) containing 0·01% NaN_3, for 30 min at 20°C. After one wash with PBS, 50 μl of serial dilutions of test sera in 3% BSA/PBS were added in duplicate to wells. Plates were incubated for 6 h at 20°C and washed four times with PBS. Subsequently, ^{125}I-labelled goat anti-rabbit immunoglobulin (GaRIg) was added (4×10^4 ct/min/50 μl/well), plates were incubated for 18 h at 4°C, washed four times with PBS, dried, and counted in a γ-counter. Binding activity of the test serum was expressed as the reciprocal serum dilution giving 50% binding (ABT_{50} titre) or as binding activity only at one particular serum dilution.

Paratope competition test (PCT). Microtitre plates were coated with 10 μg/ml MLSE for 18 h at 4°C, blocked with 3% BSA/PBS and serially diluted test sera were added at 25 μl per well in duplicate. Plates were incubated for 1 h at 20°C and ^{125}I-MoAb ($3–6 \times 10^4$ ct/min/25 μl/well) was added without prior washing. After incubation for 18 h at 4°C, plates were washed four times with PBS, dried and counted. Counts were corrected for background binding and serum titres representing reciprocal values of dilutions giving 50% inhibition (ID_{50}) of binding of the ^{125}I-MoAb to the MLSE-coated wells were calculated. Using this test, competition of binding was demonstrable with rabbit antisera (Table 1), mouse antisera (Fig. 1) or MoAb (Table 2).

Table 1. Definition of four idiotype specificities by rabbit antisera

Antigen mol.wt	MoAb code	Ig class	PCT ID_{50} titre of anti-Id serum*			
			Rb04	Rb38	Rb10	Rb06
35 kD	ML04	G1	2300	<1	<1	<1
	ML03	G1	1300	<1	<1	<1
	ML11	G1	2200	<1	<1	<1
	ML38	G1	<1	780	<1	<1
12 kD	ML10	G2a	<1	<1	2100	<1
	ML05	G1	<1	<1	3800	<1
	ML12	G1	<1	<1	3400	<1
	ML06	G1	<1	<1	<1	2500

* Dilution of rabbit anti-Id serum giving 50% inhibition of ^{125}I-MAB binding to MLSE coated plates.

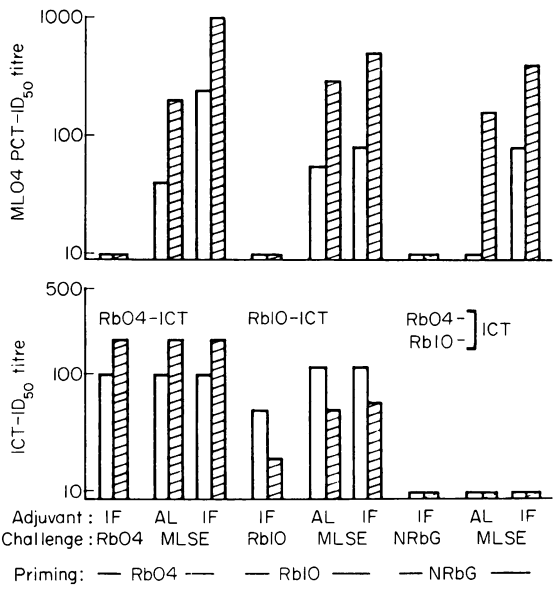

Fig. 1. Serum Id and antibody response in mice following consecutive injection with Rb anti-Id and antigen. BALB/c mice (six per group) were injected intraperitoneally with 50 μl of Rb04, Rb10 or normal rabbit globulin (NRG) emulsified in incomplete Freunds adjuvant (IF). Mice were challenged with 50 μl of homologous anti-Id (soluble) or 50 μg MLSE in either alum (AL) or IF after 2 weeks and with soluble anti-Id or MLSE, after 4 weeks. Sera bled at (□) 4 weeks and (■) 6 weeks were pooled for each group and tested for homologous idiotype (ICT assay) and ML04 paratope (PCT assay) serum ID_{50} levels.

Idiotype competition test (ICT). Microtitre plates were coated overnight at 4°C with rabbit anti-idiotype antiserum (Rb anti-Id) at an optimal dilution ranging between 1/500 to 1/3500. Subsequent procedures were the same as for the PCT assay.

Dissociative effect of Rb anti-Id on antigen-antibody complexes. Plates coated with MLSE (see PCT) were incubated with ^{125}I-MoAb for 20 h at 4°C and washed. Serially diluted anti-Id antisera were added to wells, incubated for 3 h at 20°C, washed and counted for remaining bound

Table 2. Relative affinities of paratope and idiotype binding for four MoAb

	ID_{50} ng of MoAb/well		Rb anti-Id titres	
Specificity	PCT*	ICT†	ABT_{50}‡	DT_{25}§
ML(Rb)04	8	2	12,000	90
ML(Rb)38	4	0·4	2,500	< 10
ML(Rb)10	30	0·2	10,000	1,200
ML(Rb)06	20	0·4	10,000	4,500

* Inhibition of ^{125}I-MoAb binding to MLSE plates.

† Inhibition of ^{125}I-MoAb binding to Rb anti-Id coated plates.

‡ 50% Binding of Rb anti-Id to MoAb coated plates developed by ^{125}I-Ga RIg.

§ 25% dissociation of ^{125}I-MoAb bound to MLSE coated plates by incubation for 3 h at 20°C with serially diluted homologous Rb anti-Id sera.

radioactivity. Percent dissociation was calculated in relation to wells incubated with 3% BSA/PBS instead of anti-Id serum (100%).

Immunization of mice. Inbred mice aged 6–10 weeks were purchased either from Olac Ltd, UK, or from the National Institute for Medical Research, London. Mycobacterial antigen or antiserum was prepared as an emulsion in incomplete Freund's adjuvant (IFA) (Difco) at a volume ratio of 1/1, and injected intraperitoneally. The amount of injected materials was at 50 μg MLSE or 50 μl of Rb anti-Id per animal, in a total volume of 100 μl.

Statistical analysis. Statistical significance was analysed by the Student's *t*-test, and confidence limits (P) of $< 0{\cdot}05$ were considered to be statistically significant.

RESULTS

Characterization of rabbit anti-idiotype antisera. Serially diluted rabbit antisera were initially tested for binding to plates coated with various MoAb. All absorbed Rb anti-Id antisera bound specifically only to plates coated with the immunizing MoAb, but not to plates coated with heterologous MoAb or with NMG (Praputpittaya, 1986). The association of Ids with the antigen-binding site was determined by the PCT assay and the results are presented in Table 1. Out of four monoclonal antibodies (ML06, ML10, ML05 and ML12) with overlapping paratopes, directed towards the 12 kD antigen of *M. leprae*, ML06 carried a unique idiotype whilst ML10, ML05 and ML12 were of overlapping specificity. Out of four anti-35 kD *M. leprae* antibodies (ML04, ML03, ML11 and ML38) of overlapping paratope specificity, a unique idiotype was detected on ML38 whereas ML04, ML03 and ML11 expressed overlapping idiotypes. When comparing antisera from two rabbits each of the same idiotypic specificity, similar PCT titres were observed.

The binding of ^{125}I-MoAb to Rb anti-Id coated plates (ICT) appeared eminently sensitive to inhibition by unlabelled MoAb. Idiotype specific inhibition of binding of radio-labelled MoAb with corresponding anti-Ids but not with heterologous anti-Ids fully confirmed the results obtained with the PCT analysis. The ID_{50} dose of MoAb was invariably lower in the ICT than in the PCT assay (Table 2). This disparity was particularly striking (about 100-fold) for Rb10 and Rb06, indicating higher affinity of Id-anti-Id than of paratope-antigen binding.

It was of further interest to examine the dissociative potency of Rb anti-Id antibodies, towards antigen-MoAb complexes, as an arbitrary measure of differences in the affinity of binding between

Table 3. Genetic association of the ML04 paratope and idiotype responses in Igh-congenic mouse strains

Strain	H-2	Igh	PCT	ICT	BIA
BALB.B	b	a	1951 × 1·62**	28·5 ± 1·4**	250
BALB.K	k	a	1790 × 1·78**	28·4 ± 2·6**	40
BALB.C	d	a	1010 × 2·30*	33·0 ± 6·7	4000
CBA.AK	k	d	1284 × 2·08**	33·2 ± 2·0**	10
CBA.Ca	k	j	897 × 1·94*	44·3 ± 7·8**	100
CBA.101	k	b	511 × 3·32	18·3 ± 0·7	50
B10.D2N	d	b	300 × 4·40	17·9 ± 1·9	500
CB20	d	b	236 × 2·29	13·2 ± 1·0	700

Mice were immunized i.p. with 50 μg MLSE in IFA, challenged after 2 weeks with the same antigen in saline and bled 2 weeks later.

* Geometric means × s.e. of ID_{50} serum titres for ^{125}I-ML04 binding to MLSE coated plates.

† Arithmetic means ± s.e. of % inhibition of ^{125}I-ML04 binding to Rb04 coated plates with 1/20 diluted sera.

‡ Dilution of pooled sera giving 50% binding to MLSE-coated plates.

Values compared with the mean from Igh-1^b strains (CBA.101, B10.D2N and CB20) differed at ** $P<0·01$ or * $P<0·05$ significance levels.

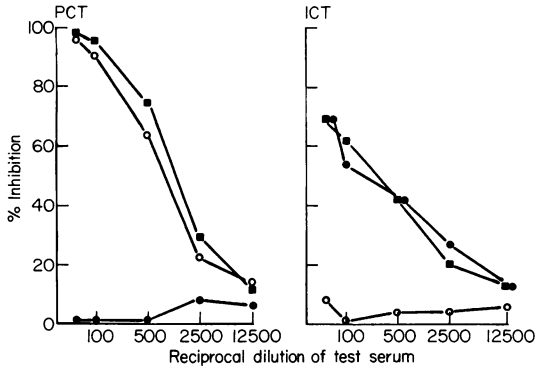

Fig. 2. Cross-absorption analysis of 'Ab3' serum. Pooled serum from BALB/c mice which had been sequentially injected with Rb04 and MLSE (see legend to Fig. 2) was passed through CNBr-Sepharose-4B columns coupled with either MLSE (●) or Rb04 (○) and tested for ML04 paratope (PCT) and idiotype (ICT) activities; non-absorbed serum (■).

the individual anti-Ids. The extent of dissociation of ^{125}I-MoAb from solid-phase bound MLSE antigen was determined following 3 h incubation at 20°C with serially diluted anti-Id sera. Results showed the strongest dissociation with Rb10 and Rb06, a less pronounced effect with Rb04 whereas Rb38 was devoid of any dissociative activity (Table 2).

Genetic control of the ML04 idiotype expression. The expression of the ML04 paratope and of the corresponding Rb04 Id within the polyclonal antibody response to MLSE was determined in allotype congenic mice (Table 3). Following immunization with MLSE in IFA, ML04-paratope titres followed a hierarchy partly associated with the Igh locus: PCT ID_{50} titres in six strains of mice

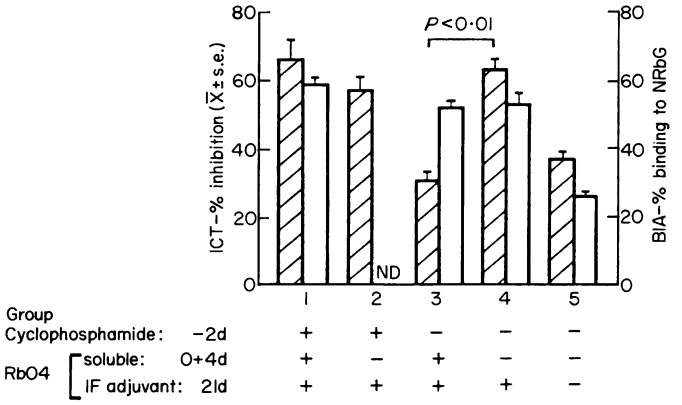

Fig. 3. Inhibition of the 'Ab3' response by soluble anti-Id serum. Relevant groups ($n=6$) of BALB/c mice were treated with 50 mg/kg cyclophosphamide, followed by 50 μl Rb04 anti-Id serum either soluble or in incomplete Freunds (IF) adjuvant. All injections were given intraperitoneally, mice were bled on day 31 and 1/20 diluted sera were tested by ICT and BIA assays (see Materials and Methods). ND, not done.

with Igh-1^a, -1^d and -1^j allotypes respectively were significantly higher than in three strains of the Igh-1^b allotype. The ML04-idiotype expression followed a similar but quantitatively less pronounced association with Igh genes. However, Igh genes did not bear any relationship to the overall antibody response to MLSE antigens, and no association was found between ML04-paratope or idiotype with H-2 haplotype.

Stimulation of serum idiotype levels by injection of anti-idiotype antibodies. Injection of Rb04 anti-Id in IFA to mice of three different strains in a preliminary experiment, showed that BALB/c mice uniformly produced the highest Id^+ levels, CBA/Ca mice produced a somewhat lower response and C57BL/6 mice, all except one, failed to respond (Praputpittaya, 1986). In the following experiment BALB/c mice were injected with either Rb04 or Rb10 anti-Id sera in IFA, followed by challenge with MLSE antigen (Fig. 1). An ICT-positive serum response to the homologous Id was found 4 and 6 weeks after injection of either Rb04 or Rb10. This response was lacking in NRbG injected mice and in respect of the unrelated idiotype specificity in sera from Rb anti-Id injected animals. Quantitatively similar ICT-ID_{50} titres were found in groups injected repeatedly with Rb anti-Id or with Rb anti-Id followed by MLSE antigen. However, even repeated injection of Rb04 failed to stimulate an antigen binding ML04 PCT-positive antibody response. The latter assay became positive only in MLSE (plus alum or IFA) immunized mice. However, the differences in ID_{50} ML04 paratope titres comparing Rb04, Rb10 or NRbG pre-injected mice were not significant. Thus, Rb04 failed to prime mice for an enhanced response to challenge with antigen.

In contrast to the ML04 paratope expression, ML10-paratope antibodies were not significantly demonstrable in any of the groups irrespective of whether the mice had been injected with MLSE with or without Rb10 anti-Id.

It was desirable to analyse the specificity of ML04 idiotype and paratope responses in sera of mice sequentially injected with Rb04 and MLSE by cross-absorbtion with either ML04 (Id) or MLSE (antigen). Results showed that the 'Ab3' serum absorbed with Rb04 was depleted only of the ML04-Id^+ but not of the ML04-$paratope^+$ molecules (Fig. 2). Conversely, the MLSE-column removed only ML04-paratope but not ML04Id^+ molecules. These results suggest that the 'Ab3' serum contained two separate antibody populations induced in response to Rb04 anti-Id and the MLSE antigen respectively.

Inhibition of the Ab3 response by soluble anti-Id serum. Injection of mice with soluble Rb04 instead of Rb04/IFA failed to stimulate a significant Ab3 (Id^+) response in several experiments (Praputpittaya, 1986). It was of further interest to investigate whether soluble Rb04 could inhibit the Id^+ response induced by subsequent injection of Rb04/IFA. BALB/c mice were injected i.p.

twice with 50 μl of Rb04 in saline, followed by an injection of 50 μl of Rb04 emulsified in IFA 1 week later. The effect of 50 mg/kg cyclophosphamide (CY) given 2 days before soluble Rb04 was also investigated. Sera bled 10 days after Rb04/IFA injection were tested at 1/20 dilution for the ML04 idiotype expression and as a specificity control, for antibodies to NRbG (Fig. 3). Sera from the positive control group of mice that had been injected with Rb04 + IFA (group 4) showed a mean 63% inhibition of binding (ICT) to Rb04 whereas mice preinjected with soluble Rb04 before Rb04 + IFA challenge (group 3) showed a significantly reduced (31% ICT) AB3-Id$^+$ response. Moreover, the suppressive effect of soluble Rb04 anti-Id was abrogated (66% ICT) in animals pretreated with CY (group 1). Results in group 2 suggested that CY itself did not inhibit the response to Rb04 + IFA alone.

Mice injected with Rb04/IFA produced also antibodies detected by binding to NRbG. However, this response was not inhibited by injection of soluble Rb04 (group 3, Fig. 3). Moreover, sequential injection of soluble and IFA-Rb04 produced no significant effect on the ML04 paratope antibody response following challenge with MLSE antigen (results not shown).

DISCUSSION

Xenogeneic rabbit antisera raised against eight murine MoAb to the 35 kD and 12 kD antigens of *M. leprae* were proved to be idiotype specific following cross-absorption with normal mouse globulin. With the exception of one specificity Rb06 which was expressed on H-chains, all other idiotypes were detected only on intact MoAb molecules. All rabbit anti-Id antibodies inhibited the binding of their corresponding MoAb to antigens with inhibitory ID_{50} titres ranging from 1/780 to 1/3800. These differences in titres are attributable mainly to antibody concentration but might also be due to differences in antibody affinities and in the topography of Id determinants in relation to the paratope of the MoAb. Although soluble antigen inhibited ML04-solid phase antigen binding, it failed to inhibit Id binding to solid-phase anti-Id (results not shown) indicating that either Id-anti-Id binding is of higher affinity than that of paratope-antigen binding, or that the Id is topographically placed in the periphery of the combining site.

Partial inhibition was reported for anti-Id/Id binding by phosphorylcholine (Sher & Cohn, 1972) and different degrees of Id association with antigen-binding sites were demonstrated for anti-(4 hydroxy-3-nitrophenyl) acetyl MoAb (Reth, Imanishi & Rajewsky, 1979). The capacity of certain rabbit anti-Ids to dissociate immune complexes between ^{125}I-labelled MoAb and solid-phase antigen confirmed that MoAb bound with higher binding affinity to anti-Ids than to antigen. Although the experimental data have yet given little information on the possible relationship between anti-Id affinity and its biological potency, these parameters might be relevant for future investigation of immune complexes in leprosy (Chakrabarty *et al.*, 1983).

The extent that MoAb with the same *M. leprae* epitope specificity share idiotypes is demonstrated by the finding that in both groups of four MoAb which recognize the 12 kD and 35 kD antigens respectively, one Id is represented on a single MoAb and the other is shared between three MoAb. Previous studies showed that expression of recurrent Ids is linked to Igh genes (Claflin, Hudak & Maddelena, 1982). Expression of the ML04 paratope and idiotype were also found to be at least partly associated with the Igh locus. In particular, low levels of both were found in Igh^b strains. However, levels of the idiotype were much lower than that of the ML04 paratope. It is conceivable that as the bulk of these antibodies were Rb04-Id negative, a very heterogeneous collection of 'private' Ids is created by V gene somatic mutations, with an overriding effect over Igh genes. Similarly in other systems, the majority of xenogeneic anti-Id sera against MoAb detect relatively private idiotypes (Bluestone *et al.*, 1986).

Immunization of mice with Rb04 or Rb10 anti-Id antisera in IFA, induced Id$^+$ 'Ab3' responses specific for the corresponding anti-Id used for the immunization. However, immunization with both anti-Id antisera failed to induce Id$^+$ antigen-binding molecules. Furthermore, the antibody paratope response of Rb04 anti-Id primed mice following challenge with MLSE antigen was not significantly increased, when compared to controls. Analysis of antibodies in sera of mice

sequentially injected with Rb04 anti-Id and MLSE antigen demonstrated two different immunoglobulin populations expressing the idiotype and paratope specificities respectively. These results suggest that despite the combining site association of the idiotypes (see competition data), neither of the two tested anti-Ids contain the functional 'internal image' of corresponding epitopes from the respective antigens. Injection of Rb04 anti-Id could have induced antibodies of several unknown paratope specificities, all expressing the Rb04 idiotype or antibodies towards the rabbit Rb04 idiotype. The latter possibility seems unlikely since a common idiotype would not be expected on polyclonal antibodies from outbred rabbits. The prevailing evidence suggests that injection of anti-Ids stimulate B cells producing immunoglobulins of homologous idiotype as the original Ab1 antibody (Takemori *et al.*, 1982). Moreover, one may speculate that competition between the stimulated Id^+ B cells with distinct paratope specificities may restrict the expression of that of the Ab1 antigen binding specificity.

Previous studies showed that injection of anti-Id antibodies can result in either enhancement or suppression of immune responses. Several factors influencing the outcome, such as the dose and isotype of anti-Id and genetic requirements of the expression of Id and/or paratope, have been reported (Eichmann & Rajewsky, 1975; Kelsoe, Reth & Rajewsky, 1980). In this paper it has been demonstrated that the form of a given anti-Id antibody plays an important regulatory role. Injection of soluble Rb04 anti-Id not only failed to stimulate but suppressed the Id^+ Ab3 response to a subsequent injection of the anti-Id in IFA. The inhibitory effect of soluble Rb04 was apparently idiotype-specific, since the anti-rabbit Ig response was not suppressed. Furthermore, the regulatory effect apparently involved a cyclophosphamide sensitive cell population. Since the stimulation of Ab3 responses (with or without internal image) by anti-Id antibodies is T cell dependent (Auchincloss *et al.*, 1983), it is conceivable that regulation of this response by CY-sensitive cells may be similar to the control of murine immune responses to protein antigens (Turk & Parker, 1982). This mechanism may also have been responsible for the initial observations of idiotype suppression following inoculation of xenogeneic anti-Id sera (Hart *et al.*, 1972). The demonstration of adjuvant dependancy of the anti-Id induced Ab3 response is in accord with other studies where the anti-Id was presented conjugated with KLH (UytdeHaag & Osterhaus, 1985) LPS or dendritic cells (Francotte & Urbaine, 1985). Finally, the observed lack of antigen binding by Id^+ Ab3 molecules is not too surprising, as this has been the common outcome also in other systems (Oudin & Cazenave, 1971; Bluestone *et al.*, 1986). Further analysis of the exceptional idiotypes which did reveal antigen specific internal image function (Rees *et al.*, 1987; Praputpittaya & Ivanyi, 1987) may identify the critical structural determinants.

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