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Recognition of *Mycobacterium leprae* recombinant 18 000 MW epitopes by IgG subclasses in leprosy

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SUMMARY

IgG subclasses are known to be differentially regulated by cytokines (elaborated by activated T cells), which act as growth factors and immunoglobulin switch factors on B cells. In leprosy, we have previously shown that IgG subclass antibodies to a purified recombinant antigen of *Mycobacterium leprae* (18 000 MW) are restricted to IgG1 and IgG3 across the disease spectrum. The only significant difference observed was that lepromatous patients with low to undetectable T-cell responses showed a strong correlation between IgG1 and IgG3 ($P < 0.001$) antibodies while tuberculoid patients who showed strong T-cell responses did not show such a correlation. To examine if these differences were related to T-cell-mediated class switching in tuberculoid leprosy patients, we have studied epitope recognition by IgG1 and IgG3 using a panel of synthetic peptides spanning the 18 000 MW molecule in an enzyme-linked immunosorbent assay (ELISA). In lepromatous patients there was little similarity in peptide recognition by IgG1 and IgG3, with IgG1 recognition being restricted to a single dominant carboxy-terminal peptide while the IgG3 antibodies recognized a diverse set of peptides in the N-terminal half of the 18 000 MW molecule. In tuberculoid patients both IgG1 and IgG3 antibody showed recognition of similar peptides in the N-terminal half of the 18 000 MW molecule. Our results therefore support the hypothesis that immunoglobulin class switching is occurring in tuberculoid but not in lepromatous patients.

INTRODUCTION

Selective activation of IgG subclasses during infection may have important implications in disease progression because of the inherent differences in the biological functions of each IgG subclass.[1,2] Cytokines elaborated by activated T cells provide both growth and immunoglobulin switch factors for B cells.[3] However, little is known about the mechanisms which control IgG subclass switching in human disease. Infection with leprosy results in polarization of T cells and humoral responses at the two ends of the leprosy spectrum, with increasing antibody responses at the lepromatous pole and increasing T-cell responses towards the tuberculoid pole.[4,5] Leprosy, therefore, provides an ideal disease to study the impact of T-cell activation on the quality of IgG subclass antibody responses. We have reported previously that the IgG subclass antibody response to the recombinant *Mycobacterium leprae* 18 000 MW antigen was restricted to IgG1 and IgG3 subclasses irrespective of the level of T-cell activation. The only significant difference observed was that lepromatous patients showed a strong correlation between IgG1 and IgG3 antibodies ($P < 0.001$) while tuberculoid patients did not show such a correlation.[6] To examine if these differences were related to differential T-cell activation in leprosy patients, we studied the *M. leprae* 18 000 MW epitopes recognized by IgG1 and IgG3 antibodies. Binding of IgG subclass antibodies to a series of overlapping 15-mer synthetic peptides spanning the entire 18 000 MW molecule was analysed. Tuberculoid patients showed recognition of several similar peptides by both IgG1 and IgG3, but in the case of lepromatous patients IgG3 and IgG1 showed distinct patterns of peptide recognition. Our results are, therefore, consistent with the hypothesis that downstream class switching from IgG3 to IgG1 and maturation of the antibody response is not occurring in patients with lepromatous leprosy but is occurring in patients with tuberculoid leprosy.

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MATERIALS AND METHODS

Leprosy patients

Untreated leprosy patients presenting at The Marie Adelaide Leprosy Center, Karachi, Pakistan, and without any evidence of reactional complications were recruited to the study. Patients were diagnosed clinically as well as histologically on a 4-mm

punch biopsy taken from the edge of an active lesion.[7] Sera from 19 lepromatous and borderline lepromatous (LL/BL) designated as L, and nine tuberculoid and borderline tuberculoid (BT/TT) designated as T, leprosy patients were selected for epitope mapping based on the *M. leprae* 18 000 MW positivity with IgG1 and IgG3 antibodies. Negative controls included a serum pool from five healthy endemic donors; sera from two leprosy patients that were negative for IgG1 and IgG3 antibodies to 18 000 MW were also included as controls for high levels of irrelevant antibodies.

Antisera

Five millilitres of blood collected from either leprosy patients or healthy endemic donors was allowed to separate overnight at 4°. Serum was removed and centrifuged at 400 *g* for 15 min; the clear supernate was distributed in small aliquots and frozen at −70° before use.

M. leprae *18 000 MW antigen*

The recombinant *M. leprae* 18 000 MW preparation used in the study was the full-length 18 000 MW kindly provided by Dr J. Watson (University of Auckland, New Zealand).[8] The antigen was *Escherichia coli* derived, ammonium sulphate precipitated and further subjected to high-performance liquid chromatography (HPLC). The protein was >95% pure as assessed by sodium dodecyl sulphate–polyacrylamide gel (SDS PAGE) electrophoresis (R. Prestidge, personal communication).

Peptide synthesis

Overlapping peptides spanning the whole length of the 18 000 MW protein were synthesized by manual solid-phase synthesis (Ramps; Du Pont, Stevenage, UK) using Fmoc chemistry on Rink resin (4–(2′, 4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin; Calbiochem, Nottingham, UK). Fmoc-protected amino acids (Bachem, Bubendorf, Switzerland) were converted to hydroxybenzotriazole-activated esters by treatment with hydroxybenzotriazole and N, N′-diisopropylcarbodiimide in dimethyl formamide (DMF). Subsequent coupling reactions were performed in DMF and the Fmoc groups were removed with 50% piperidine in DMF followed by a series of washes in DMF. After synthesis, side-chain protecting groups were removed and the peptides were cleaved off the resin in trifluoroacetic acid in the presence of appropriate scavengers. After cleavage, peptides were precipitated with diethylether and their purity assessed by analytical HPLC. Fifteen peptides (designated A–O according to the order of synthesis and not according to amino acid order) were synthesized. The peptides were 15-mer overlapping by five amino acids with two exceptions: one being the C-terminal peptide K (amino acids 131–148) and the second being peptide D (amino acids 38–50).

Reagents, monoclonal antibodies and conjugates

The monoclonal antibodies specific for human IgG subclasses used in this study were HP 6069 (anti-IgG1), kindly provided by Dr R. Hamilton (John Hopkins University, Baltimore, MD), and HP 6047 (anti-IgG3), prepared at the Center for Disease Control (Atlanta, GA; a gift from Dr C. Reimer). The specificity evaluation and performance characteristics of these antibodies in enzyme-linked immunosorbent assay (ELISA) systems have been described in detail elsewhere.[9,10] Goat anti-mouse IgG conjugated to alkaline phosphatase was obtained commercially (Jackson Laboratories, West Grove, PA) and used according to the manufacturer's recommendations.

IgG subclass antibodies to 18 000 MW and its 15-mer peptides

Immulon 4 plates were coated with 0·1 μg/well of *M. leprae* 18 000 MW or 20 μg/well of the individual peptides in carbonate buffer, pH 9·6, for 2 hr at 37° and then overnight at 4°. The plates were blocked with 200 μl phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA) for 2 hr at 37° and subsequently washed in PBS containing 0·05% Tween-20 (PBS–Tween) three times. One hundred microlitres of sera diluted in PBS–Tween containing 1·0% BSA was added to both peptide-coated and uncoated blocked wells (serum blank) at a single dilution of 1/20, and incubated for a further 2 hr at 37° and then overnight at 4°C. For detection of IgG subclass-specific antibodies, wells were incubated with monoclonal antibodies specific for IgG1 (HP 6069) or IgG3 (HP 6047) subclasses for 2 hr at 37° and then overnight at 4°. The final incubation was with anti-mouse IgG conjugated to alkaline phosphatase (Fc specific; Jackson Laboratories). A serum pool from five healthy endemic donors and IgG 18 000 MW-positive and -negative sera from leprosy patients was run in each assay to control for background binding to peptides, non-specific binding due to the presence of high titres of non-relevant antibodies in leprosy sera, and interassay variability.

18 000 MW-peptide inhibition ELISA

Peptide mixtures used for inhibition of binding of 18 000 MW antibodies were L and M (amino acids 1–15 and amino acids 11–25) or A and K (amino acids 121–135 and 131–148). Immulon 4 plates were coated with 0·1 μg/well of *M. leprae* 18 000 MW. The plates were blocked with 200 μl PBS containing 5% BSA for 2 hr at 37° and subsequently washed in PBS three times. Two high-titred sera from lepromatous leprosy patients (L607 and L973) were preincubated in triplicate at a dilution of 1 : 400 for IgG1 and 1 : 40 for IgG3 with soluble peptides L and M or A and K at a final concentration of 5 μg/ml, or 18 000 MW at 2·5 μg/ml diluted in antibody diluent containing 1% BSA in PBS with Tween-20 (0·05%), and incubated at 37° for 2 hr and subsequently at 4° overnight. One hundred microlitres of serum–peptide mixture, or control serum without peptides but treated in a similar fashion, was added to the 18 000 MW-coated plate, incubated for 2 hr at 37° and then overnight at 4°. The remaining steps of addition of IgG subclass-specific monoclonal antibodies and revealing probe were similar to that described above for the IgG subclass ELISA. Percentage inhibtion was calculated as percentage reduction in binding compared to control serum.

Statistical analysis

Student's *t*-test was used to assess the significance of differences in inhibition of IgG1 and IgG3 binding after preincubation with soluble peptides in patients' sera.

RESULTS

Characterization of sera for epitope mapping

A panel of 28 sera (19 LL, five BT and four TT) with defined

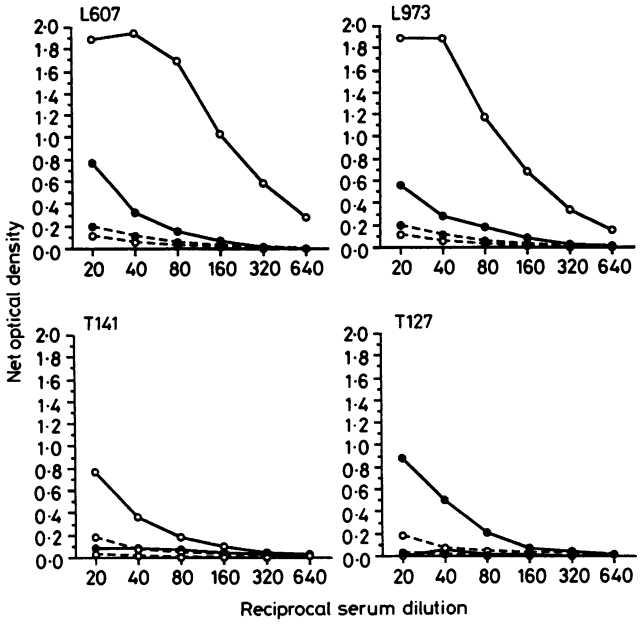

Figure 1. Dilution curves in selected sera from patients with lepromatous leprosy (L) and tuberculoid (T) disease. Solid lines indicate patients' sera. Broken lines are pooled sera from healthy endemic donors. Open circles indicate IgG1 and closed circles IgG3 antibody responses to *M. leprae* 18 000 MW.

antibody responses was selected for use in the peptide-binding experiments after screening 127 untreated leprosy patients. Each serum was titrated to determine the concentration of IgG1 and IgG3 anti-18 000 MW antibodies. Sera were selected for strong 18 000 MW recognition by either the IgG1 or IgG3 subclass antibody. Examples of dose–response curves and selective antibody subclass responses are shown in Fig. 1. The inclusion of sera containing antibodies belonging to a single subclass provided controls for competition among subclasses of antibodies that could mask responses to certain common epitopes.

Peptide binding by IgG1 and IgG3 antibodies

Binding of peptides by IgG1 antibodies. The results obtained with the individual sera from leprosy patients are given in Table 1 for IgG1 antibody responses to the peptides. A panel of 15 overlapping peptides spanning the sequence of the *M. leprae* 18 000 MW antigen was tested for binding with antibodies at a single dilution (1 : 20) of patients' serum. Sera are ranked in the table according to the titre of the relevant IgG subclass. Sera from two leprosy patients without detectable antibodies (titre < 20) in either subclass were included as controls for non-specific binding to peptides due to the high titres of non-relevant antibodies reported in leprosy sera. A serum pool from healthy endemic donors was also included as a control for baseline antibody activity. The optical density obtained after deducting the control reading with the corresponding reagent blank of each individual peptide is included in the table. Because of the wide variation in non-specific binding of sera to the uncoated wells, the optical density obtained with uncoated blocked wells (serum blanks) is also indicated. Leprosy sera containing antibodies to 18 000 MW selectively in IgG1 or IgG3 subclasses provided additional reciprocal controls for each of the IgG subclasses. The corresponding reagent blank with individual peptides did not show much variation and ranged between 0·05–0·1. Corresponding reagent blank readings for each peptide were, however, deducted from the test reading. Surprisingly, individual sera showed a wide variation in non-specific binding to uncoated wells, which was observed to be much higher for IgG3 than IgG1, although the antibody titres for IgG3 were lower than IgG1 antibodies. This was, therefore, not due to non-specific factors in the serum but may be related to the structural properties of IgG3 antibodies, such as its 13–15 disulphide bonds in the hinge region making it prone to denaturation and stickiness. It was therefore neccessary to take into consideration this non-specific binding for each serum when evaluating the positive signal. The signals were considered weak if the ratio of binding with peptide-coated wells was two- to fourfold higher compared to serum blanks, and strong if the signals were $>$ fourfold higher. Optical densities below 0·1 were considered negative even if the ratio of binding was higher than 2.

It was reassuring to note that sera from lepromatous patients negative for IgG1 18 000 MW antibodies were also negative with all the peptides, indicating that there was very little background noise due to non-relevant antibodies. IgG1 in lepromatous patients showed consistent recognition of a single carboxy-terminal peptide K (amino acids 131–148), giving a signal in 11/16 IgG1 18 000 MW-positive sera, with the majority of sera showing strong signals. Five additional peptides were recognized but at the most by two lepromatous sera. Two sera (nos L838 and L672) with IgG1 18 000 MW titres of 1280 and 160 either bound very weakly or did not bind to any of the peptides. This was not due to competition with IgG3 antibodies, as both these sera had low to undetectable levels of IgG3 antibody. It is likely that antibodies in these sera may be recognizing a conformational epitope which may be lost in the linear sequence. The strongest binding was observed with sera with higher antibody titres. Not unexpectedly, in patients with tuberculoid leprosy the overall titres of IgG1 antibodies were lower than in lepromatous patients and, therefore, resulted in overall weaker signals with peptides as well. However, the peptides recognized in tuberculoid sera with IgG1 antibody were quite different than those recognized by lepromatous patients. In addition, a larger panel of peptides (L, N and I) was recognized in a more consistent fashion. Peptides L and N were recognized by 5/8 and peptide I was recognized by 7/8 IgG1-positive sera. The carboxy-terminal peptide strongly recognized by lepromatous patients showed no significant binding with tuberculoid patients. None of the peptides showed IgG1 positivity with the endemic serum pool or a tuberculoid serum with a < 20 IgG1 anti-18 000 MW antibody titre, indicating that the peptides were being recognized in a specific fashion.

Binding of peptides by IgG3 antibodies. The results obtained with IgG3 antibodies are given in Table 2. The most striking observation was that, unlike IgG1, IgG3 showed a very similar pattern of peptide recognition in both lepromatous and tuberculoid patients. Surprisingly, IgG3, despite an overall lower titre than IgG1 to 18 000 MW, showed a much broader pattern of recognition than IgG1 subclass antibodies in both

Table 1. Binding of IgG1 antibodies to synthetic peptides spanning the *M. leprae* 18 000 MW molecule

Amino acids			1–15	11–25	21–35	31–45	38–50	41–55	51–65	61–75	71–85	80–94	90–105	101–115	111–125	121–135	131–148
Donor*	IgG1 titre	Serum blank	L	M	N	O	D	E	F	G	B	C	H	I	J	A	K
L607	1280	0·147	0·197	0·171	0·261	0·162	0·151	0·169	0·241	0·247	0·102	0·092	0·168	0·225	0·137	0·785	>2·0
L838	1280	0·100	0·100	0·090	0·088	0·168	0·090	0·070	0·070	0·040	0·060	0·060	0·063	0·127	0·054	0·217	0·206
L973	640	0·207	0·292	0·247	0·326	0·224	0·273	0·237	0·288	0·215	0·160	0·144	0·171	0·296	0·218	0·193	1·320
L628	320	0·090	0·110	0·108	0·137	0·158	0·098	0·086	0·085	0·097	0·089	0·062	0·066	0·129	0·074	0·153	>2·0
L961	320	0·239	0·321	0·323	0·419	0·357	0·292	0·235	0·394	0·250	0·301	0·207	0·205	0·377	0·206	0·142	1·307
L193	160	0·134	0·191	0·138	0·211	0·137	0·177	0·218	0·170	0·127	0·096	0·063	0·144	0·212	0·097	0·083	1·554
L710	160	0·136	0·143	0·161	0·206	0·154	0·139	0·115	0·141	0·149	0·169	0·112	0·110	0·223	0·127	0·130	1·208
L735	160	0·136	0·125	0·163	0·123	0·762	0·347	0·263	0·238	0·122	0·115	0·072	0·151	0·115	0·076	0·110	0·322
L974	160	0·115	0·131	0·127	0·132	0·191	0·169	0·131	0·142	0·098	0·115	0·099	0·100	0·162	0·341	0·062	0·099
L672	160	0·020	0·039	0·015	0·022	0·067	0·005	0·008	0·005	0·014	0·011	0·019	0·008	0·017	0·007	0·014	0·018
L808	40	0·297	0·464	0·318	0·546	0·109	0·328	0·273	0·436	0·476	0·302	0·285	0·467	0·501	>2·0	0·297	0·808
L604	20	0·075	0·089	0·034	0·054	0·008	0·069	0·068	0·029	0·026	0·040	0·050	0·050	0·076	0·029	0·060	0·520
L650	20	0·148	0·173	0·126	0·194	0·161	0·137	0·493	0·168	0·047	0·092	0·134	0·131	0·111	0·113	0·059	0·173
L790	20	0·314	0·519	0·453	0·386	0·177	0·403	0·620	0·510	0·677	0·468	0·275	0·579	0·278	0·206	0·343	0·961
L831	20	0·100	0·095	0·122	0·119	0·000	0·108	0·112	0·117	0·080	0·096	0·145	0·076	0·114	0·065	0·083	0·052
L957	20	0·107	0·169	0·083	0·087	0·083	0·130	0·137	0·138	0·114	0·102	0·114	0·079	0·225	0·109	0·174	0·009
L745	<20	0·050	0·083	0·057	0·028	0·059	0·011	0·060	0·063	0·090	0·080	0·078	0·044	0·066	0·049	0·091	0·045
T130	320	0·070	0·090	0·089	0·137	0·075	0·046	0·030	>2·0	0·052	0·073	0·058	0·056	0·166	0·109	0·051	0·073
T083	80	0·279	0·727	0·368	0·903	0·077	0·355	0·693	0·375	0·338	0·323	0·203	0·482	0·558	0·126	0·232	0·290
T141	80	0·350	0·770	0·586	0·878	0·391	0·711	0·442	0·663	0·584	0·511	0·403	0·522	0·700	0·515	0·580	0·038
T029	40	0·296	0·735	0·332	1·035	0·083	0·313	0·426	0·382	0·361	0·098	0·448	0·534	0·793	0·379	0·339	0·406
T336	40	0·150	0·323	0·214	0·338	0·256	0·296	0·203	0·286	0·214	0·145	0·077	0·255	0·323	0·191	0·127	0·115
T102	40	0·075	0·117	0·116	0·102	0·024	0·042	0·125	0·082	0·117	0·062	0·033	0·139	0·188	0·068	0·051	0·067
T100	40	0·180	0·184	0·181	0·231	0·010	0·127	0·181	0·168	0·256	0·144	0·127	0·977	0·387	0·200	0·153	0·123
T127	40	0·070	0·165	0·131	0·191	0·116	0·118	0·102	0·135	0·047	0·106	0·044	0·072	0·112	0·028	0·036	0·115
T1019	<20	0·058	0·050	0·029	0·050	0·021	0·020	0·054	0·039	0·046	0·037	0·014	0·024	0·047	0·045	0·031	0·032
EC3	<20	0·155	0·184	0·151	0·197	0·156	0·168	0·191	0·092	0·151	0·116	0·093	0·149	0·065	0·200	0·137	0·155

*L indicates lepromatous and T tuberculoid leprosy patients.
signal/noise ratio: 2–4 is shown in ; >4 is shown in .

Table 2. Binding of IgG3 antibodies to synthetic peptides spanning the *M. leprae* 18 000 MW molecule

Amino acids			1–15	11–25	21–35	31–45	38–50	41–55	51–65	61–75	71–85	80–94	90–105	101–115	111–125	121–135	131–148
Donor*	IgG3 titre	Serum blank	L	M	N	O	D	E	F	G	B	C	H	I	J	A	K
L628	640	0·250	0·223	0·290	0·220	1·352	0·882	0·838	0·824	0·462	0·532	0·126	0·491	0·152	0·043	0·063	0·358
L808	320	0·259	0·524	0·366	0·556	0·414	0·485	0·570	0·410	0·359	0·357	0·284	0·246	0·402	0·248	0·376	>2·0
L650	160	0·527	1·720	1·019	1·920	>2·0	>2·0	>2·0	1·440	0·531	0·738	0·451	0·534	0·780	0·178	0·156	0·593
L831	160	0·143	0·730	0·198	0·426	0·559	0·358	0·732	0·280	0·037	0·113	0·084	0·080	0·231	0·057	0·051	0·144
L604	160	0·700	1·545	1·244	1·375	1·242	1·527	1·813	1·380	1·120	1·040	1·030	1·270	1·240	0·330	0·730	1·080
L973	80	0·196	0·279	0·160	0·288	0·520	0·466	0·717	0·397	0·212	0·236	0·172	0·314	0·270	0·077	0·000	0·116
L607	80	0·207	0·598	0·335	0·472	0·965	1·032	1·635	0·638	0·304	0·267	0·173	0·525	0·284	0·028	0·104	0·958
L957	40	0·360	1·559	0·646	1·044	1·838	1·350	1·693	0·772	0·299	0·460	0·318	0·406	0·566	0·190	0·245	0·428
L609	40	0·591	0·674	0·783	0·645	>2·0	1·318	1·439	1·073	0·677	0·749	0·400	1·037	0·325	0·125	0·149	0·591
L961	40	0·524	1·288	0·846	1·277	0·900	1·150	1·360	1·019	0·695	0·524	0·718	0·979	0·924	0·387	0·655	0·837
L710	40	0·470	1·040	0·847	1·070	0·990	0·820	0·992	0·960	0·762	0·630	0·590	0·840	0·750	0·320	0·617	0·744
L735	40	0·200	0·760	0·260	0·760	0·860	0·530	0·450	0·460	0·213	0·060	0·170	0·290	0·760	0·000	0·050	0·010
L790	40	0·126	0·350	0·202	0·330	0·300	0·290	0·303	0·195	0·162	0·170	0·198	0·213	0·287	0·106	0·130	0·159
L733	20	0·106	0·211	0·088	0·174	1·077	0·934	0·254	0·414	0·110	0·096	0·114	0·128	0·249	0·062	0·086	0·078
L193	<20	0·230	0·570	0·252	0·460	0·494	0·780	0·670	0·396	0·268	0·368	0·384	0·357	0·300	0·120	0·270	0·220
L672	<20	0·040	0·040	0·023	0·048	0·040	0·040	0·061	0·050	0·029	0·011	0·008	0·028	0·023	0·018	0·020	0·008
T102	80	0·105	0·650	0·189	0·601	0·220	0·402	0·581	0·230	0·030	0·050	0·105	0·130	0·210	0·000	0·017	0·030
T127	80	0·523	0·658	0·501	0·704	0·371	0·776	0·756	0·664	0·613	0·612	0·752	0·633	0·601	0·487	0·995	0·553
T029	40	0·060	0·680	0·102	0·782	0·291	0·339	0·530	0·180	0·010	0·000	0·000	0·017	0·309	0·000	0·000	0·000
T083	40	0·070	0·474	0·176	0·337	0·000	0·198	0·397	0·000	0·000	0·138	0·040	0·060	0·000	0·000	0·925	0·000
T130	20	0·132	0·161	0·129	0·158	0·341	0·328	0·171	0·251	0·146	0·148	0·117	0·177	0·201	0·095	0·139	0·090
T141	20	0·006	0·024	0·005	0·008	0·005	0·000	0·007	0·000	0·005	0·003	0·009	0·000	0·000	0·000	0·001	0·002
T336	<20	0·073	0·120	0·050	0·095	0·107	0·160	0·134	0·100	0·070	0·084	0·073	0·080	0·090	0·036	0·074	0·049
T1019	<20	0·030	0·008	0·003	0·004	0·004	0·009	0·031	0·012	0·010	0·010	0·000	0·007	0·000	0·000	0·011	0·004
T100	<20	0·050	0·134	0·040	0·152	0·020	0·060	0·118	0·050	0·020	0·010	0·020	0·040	0·050	0·000	0·010	0·020
EC3	<20	0·062	0·110	0·046	0·091	0·084	0·132	0·161	0·098	0·063	0·058	0·059	0·090	0·088	0·040	0·062	0·040

*L indicates lepromatous and T tuberculoid leprosy patients.
Signal/noise ratio: 2–4 is shown in ; >4 is shown in .

Table 3. Specificity of antibody binding to peptides

Serum	Treatment*	IgG1† OD (% inhibition)	IgG3† OD (% inhibition)
L973	None	0·447(0·0)	0·279(0·0)
	18 000 MW	0·038(92)	0·106(62)
	L and M (amino acids 1–25)	0·403(10)	0·280(0·0)
	A and K (amino acids 121–148)	0·239(47)	0·272(0·0)
L607	None	1·112(0·0)	0·448(0·0)
	18 000 MW	0·076(77)	0·240(47)
	L and M (amino acids 1–25)	1·056(5·0)	0·171(62)
	A and K (amino acids 121–148)	0·460(55)	0·070(84)

*Sera were preincubated with 18 000 MW at final concentration of 2·5 μg/ml or with peptide mixtures at 5·0 μg/ml or with diluent alone as control.

†Sera were tested in triplicate at a final concentration of 1/400 for binding of IgG1 antibody at 18 000 MW and a 1/40 for binding of IgG3. Results are expressed as means of the triplicate and percentage inhibition compared to control values.

lepromatous and tuberculoid patients. The main region recognised by IgG3 antibodies was the N-terminal half of the 18 000 MW molecule. Five peptides strongly recognized by both lepromatous and tuberculoid patients' sera were L, N, O, D and E; all these peptides were also recognized by IgG1 in tuberculoid sera. The carboxy-terminal peptide K (amino acids 131–148), strongly recognized by IgG1 in lepromatous patients, bound IgG3 in only 2/14 sera tested. The healthy

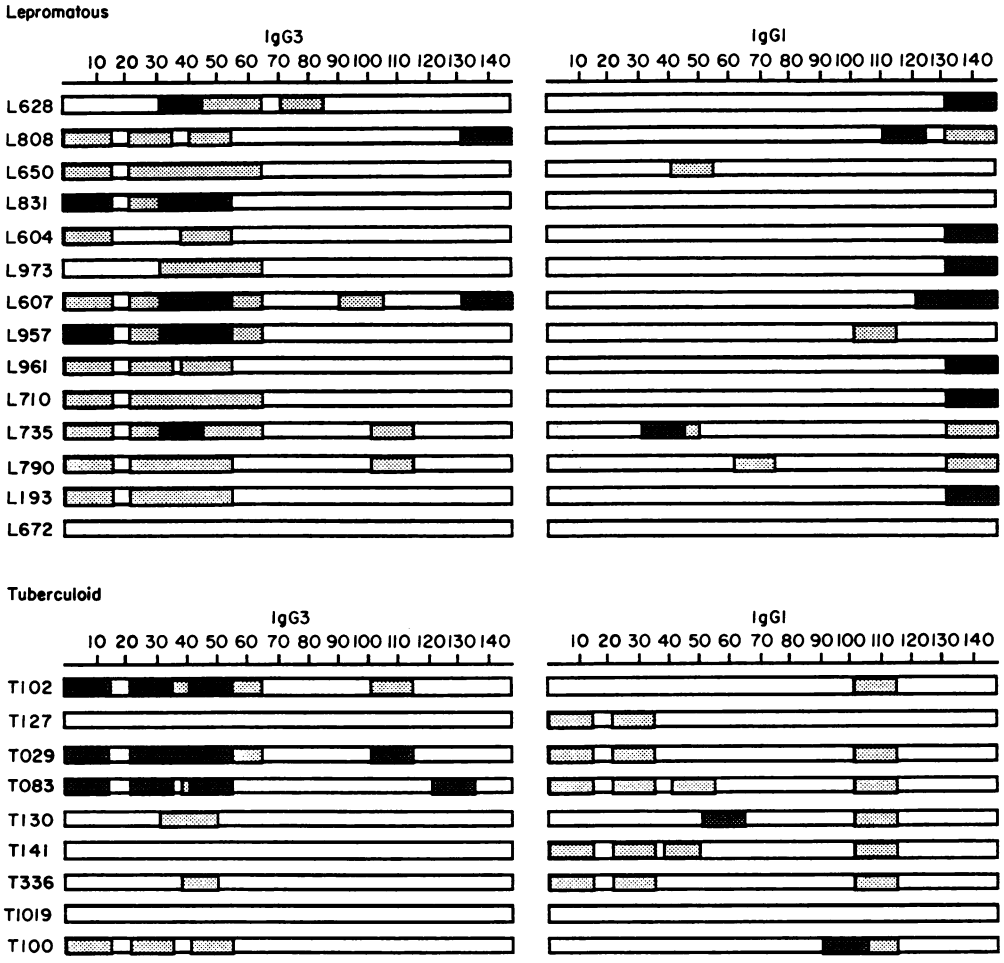

Figure 2. Comparative binding of IgG3 and IgG1 in individual patients. Darker boxes indicates strong binding (signal/noise ratio >4·0) and lighter boxes indicate weak binding (signal/noise ratio 2–4).

endemic serum pool bound two of the peptides, peptides D and E, weakly. Even sera with a titre of <20 to the whole 18 000 MW molecule showed weak binding with some of the peptides in the same region, indicating that even low levels of antibodies were able to give a positive signal with these peptides. Thus binding of peptides with IgG3 antibodies was similar to IgG1 antibodies in tuberculoid patients but not in lepromatous leprosy patients.

Specificity of antibody binding to the peptides

The specificity of antibody binding to peptides was evident by an absence of binding with sera from (1) healthy endemic donors and (2) leprosy patients with no detectable antibody to 18 000 MW. However, to confirm further the specificity of peptide binding to antibodies in the leprosy sera, antibody subclass binding to 18 000 MW on the plastic surface was inhibited with soluble peptides. The binding of IgG1 and IgG3 antibodies to 18 000 MW was inhibited in sera of two patients (L607 and L973) with two sets of peptide mixtures: L and M containing the amino-terminal peptides (amino acids 1–15 and 11–25) and A and K containing the carboxy-terminal peptides (amino acids 121–135 and 131–148). For IgG1 inhibition a dilution of 1 : 400, and for IgG3 a dilution of 1 : 40, was used, which corresponded to the mid-range of the binding curve with 18 000 MW (Fig. 1). The percentage reduction in binding for IgG1 and IgG3 is shown in Table 3. Patient L973, who had shown strong recognition of peptides A and K with IgG1 (Table 1) showed 47% inhibition ($P < 0{\cdot}0001$). Peptide mixture L and M, which had given a signal lower than our cut-off point for positivity (Table 2), also showed lower (10%) but significant ($P < 0{\cdot}09$) inhibition of IgG1 binding in L973. However, both peptides showed no inhibition in IgG3-binding assays for L973, which also did not recognize these peptides in peptide-binding assays, indicating that the signals in the peptide-binding assay were highly specific.

Similarly, IgG1 antibodies in L607 showed 55% inhibition with A and K and 5% inhibition with peptide mixture L and M, again corresponding to the antibody signals obtained in the peptide binding. The IgG3 antibodies in L607 were inhibited to a similar degree by both peptides ($P < 0{\cdot}001$). Again, as expected, 18 000 MW showed significant ($P < 0{\cdot}0001$) inhibition with both IgG1 and IgG3 in L607. These results further substantiate the IgG1 and IgG3 antibody binding observed to these peptides and confirm that the signals observed with the peptides (Tables 1 and 2) were highly specific. IgG1 in both sera showed much higher inhibition than IgG3 antibodies. One explanation may be that the IgG1 antibodies are of higher affinity than the IgG3 antibodies and therefore more inhibitable. The differences in epitope recognition by IgG1 and IgG3 is much more clearly appreciated when the two polar groups are compared simultaneously (Fig. 2). Similar peptide recognition in tuberculoid but not in lepromatous patients supports the hypothesis that downstream switching from IgG3 to IgG1 may be occurring in tuberculoid patients but not in patients with lepromatous leprosy.

DISCUSSION

In order to analyse the impact of the presence or absence of T-cell activation on epitope recognition by IgG subclasses, a panel of leprosy sera from the opposite poles of the disease spectrum was tested for their ability to bind to 15-mer synthetic peptides spanning the entire *M. leprae* 18 000 MW molecule. The antibody ELISA developed to address this issue showed specific binding with minimum background noise.

The most striking finding in these studies was the dissimilarity in peptide recognition by IgG1 and IgG3 antibodies in patients with lepromatous leprosy, although most lepromatous patients showed concurrent IgG1 and IgG3 responses, confirming our earlier finding of a strong correlation between IgG1 and IgG3 anti-18 000 MW responses in patients with lepromatous leprosy.[6] In contrast, patients with tuberculoid leprosy showed similar patterns of recognition with both IgG1 and IgG3. The immunoglobulin gene arrangement on human chromosome 14 is μ-δ-γ_3-γ_1 constant genes.[11] Interferon-γ produced by the T-helper type 1 (Th1) subset is the cytokine that up-regulates murine IgG2a (homologous to human IgG3) synthesis in lipopolysaccharide (LPS)-activated B cells.[3] The switching of the IgM response to IgG3 and IgG1 occurs in patients with lepromatous leprosy without detectable Th1 cell responses, which are usually considered to be the source of interferon-γ. However, this cytokine is also derived from cell sources other than the Th1 subset, such as the δT cells,[12] which are selectively activated by mycobacterial antigens[13] and natural killer (NK)[14] cells. Interferon-γ from these sources may, therefore, initiate IgG3 and IgG1 synthesis in the absence of T-cell help. It is believed that intermediate double-producer stages of B cells exist, where the new isotype is first synthesized from alternative splicing of multi-CH RNA transcripts.[15,16] This cell is postulated to be the target of additional factors for downstream gene switching. It is unclear if the same factors which activate the B cells to synthesize the multi-CH RNA transcripts also participate in deletion of the genes for class switching. Our results would suggest that the synthesis of IgG3 and IgG1 takes places in two stages, with the first step requiring synthesis of a multi CH-transcript which is taking place in lepromatous patients, but the second step requiring gene deletion may be absent, suggesting that additional factors for maturation of isotype responses may be absent in this group of patients but present in patients from the tuberculoid pole. Direct confirmation, however, would require the analysis of V–D–J sequence usage by different IgG subclasses in both groups of patients.

The only peptide recognized in lepromatous as well as tuberculoid patients in a consistent fashion was the carboxy-terminal peptide K (amino acids 131–148). The binding of this peptide to antibodies was highly specific, since the soluble peptide could inhibit binding of IgG1 antibody to the 18 000 MW molecule in a highly specific manner. Surprisingly, this peptide was not detected as a B-cell epitope in mice immunized with either *M. leprae*,[17] 18 000 MW[18] or peptide amino acids 131–148,[19] although we have recently produced several mouse monoclonal antibodies to the *M. leprae* 18 000 MW molecule that recognize this peptide (B. Menz, unpublished results).

The dominant L5 epitope (109–115) described using mouse monoclonal antibodies[17] and shown to be *M. leprae* specific,[20] with *M. habana* being the sole exception,[21] showed an interesting difference in leprosy patients. In lepromatous patients the peptide containing the L5 epitope showed very little binding by either IgG1 or IgG3 antibodies. On the other hand, tuberculoid patients showed strong recognition of this

peptide by IgG1. Another L5 adjacent epitope, 116–120, recognized by mouse T-cell proliferative responses[18] was not recognized by either IgG1 or IgG3 in tuberculoid or lepromatous patients. While it is not surprising that during progressive infection, immune responses are quite different than following immunization with adjuvants in the mouse model, this point is often overlooked. Parallels between mouse and human responses should, therefore, be drawn with caution when defining epitopes such as for second generation vaccines.

In conclusion, our studies show that while subclasses of IgG antibodies induced in leprosy are not affected by differential T-cell activation, the number and nature of B-cell epitopes recognized by IgG1 are highly influenced by the presence of activated Th1 cells, and that T-cell signals which determine the regulation of human IgG subclass responses need to be identified further.

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