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Human Monoclonal Antibodies Against *Mycobacterium leprae*

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Human hybridomas were constructed which produce antibodies against three different extracts of *Mycobacterium leprae*. A thioguanine-resistant (Thg^r), ouabain-resistant (Oua^r), human lymphoblastoid cell line, KR-4, was hybridized with Epstein-Barr virus-transformed cell lines from lepromatous leprosy patients with fusion frequencies of $>10^{-5}$. Non-Epstein-Barr virus-transformed donor cells fused at much lower rates ($<2 \times 10^{-7}$). Hybrids were selected in medium containing hypoxanthine aminopterin thymidine and 10^{-5} M ouabain. An enzyme-linked immunosorbent assay was used to screen for antibodies against three crude extracts of armadillo-derived *M. leprae*, including (i) a soluble sonic extract preparation, (ii) sodium dodecyl sulfate extract of insoluble sonicated *M. leprae*, and (iii) a purified phenolic glycolipid antigen. Of a total of 2,200 final clones screened, 359 were found to secrete antibody which bound to soluble sonic extracts and the sodium dodecyl sulfate extract (6.7 and 9.6%, respectively), whereas 12.5% (21 out of 168) showed positivity to the glycolipid antigen. Four selected hybridomas also reacted with the deacylated derivative of *M. leprae* phenolic-glycolipid antigen. The specificity of these monoclonal antibodies was partially determined by screening on a panel of crude extracts from four other mycobacteria. Nine clones of 122 showed reactivity to *M. leprae* only. The predominant immunoglobulin was immunoglobulin M, and quantities up to 10 μg/ml were produced. Antibody production by hybrid clones was stable in more than 75% of the clones grown in continuous culture. By comparison, 10,000 Epstein-Barr virus-transformed lymphocyte clones from lepromatous leprosy patients were screened for anti-*M. leprae* antibody production, and all of the 42 clones that were initially positive in the enzyme-linked immunosorbent assay lost their antibody-producing capabilities within 6 weeks in culture. These results suggest that a combination of Epstein-Barr virus transformation and hybridization may be an optimal method in producing human monoclonal antibodies from leprosy patients.

Antigenic analysis of *Mycobacterium leprae*, the poorly understood causative agent of leprosy, has proceeded rapidly in recent years with the advent of armadillo-grown bacilli (19). Rabbit antisera revealed seven distinct components in sonic extracts of *M. leprae*, but all seven cross-reacted with other species of mycobacteria, as revealed by crossed-immunoelectrophoresis (9). Others have uncovered protein antigens such as 21B (21), antigen 7 (11), 12,000- and 33,000-molecular-weight glycoproteins (5) and uncharacterized proteins (1, 4) which have incomplete levels of *M. leprae* specificity. The hybridoma technique of Kohler and Milstein (16) has produced much more specific monoclonal antibody probes than heterologous antisera. Two monoclonal antibodies have been produced which react specifically with unidentified components of *M. leprae* (8) and another recognizes a 12,000-molecular-weight protein (15). Perhaps those of greatest interest recognize the terminal saccharide (3,6-di-*O*-methyl glucose) in a phenolic glycolipid of *M. leprae* (30). These monoclonal antibodies are *M. leprae* specific and do not react with similar structures from other species such as *M. kansasii*. Others have previously shown that *M. leprae* contains in its surface capsule, a unique carbohydrate portion consisting of a terminal 3,6-di-*O*-methyl glucose linked β 1→4 to 2,3-di-*O*-methyl rhamnose which is linked α 1→2 to 3-*O*-methyl rhamnose (13, 14). Sera from patients infected with *M. leprae* but not other mycobacterial species also react with this antigen in a disease- and stage-specific manner (2, 3, 6, 23, 29).

Murine monoclonal antibodies have been valuable molecular probes in these studies, but human hybridomas offer several advantages. First, the construction of human hybridomas will allow development of an antigenic map of *M. leprae*, as recognized by the human, rather than the murine, immune specificity repertoire. It is conceivable that the human immune system may recognize some novel antigenic determinants since patients, unlike mice, are infected with living, metabolizing organisms. Second, lepromatous leprosy (LL) patients are highly immune and this circumvents the need for using large quantities of a limited *M. leprae* stock in immunizing mice. Third, specific human monoclonal immunoglobulin M (IgM) might enhance the efficacy of the armadillo leprosy vaccine since similar monoclonal antibodies in mice potentiated both the primary and memory response to malaria vaccine, as revealed by enhanced survival after a lethal challange infection (12). Fourth, the production of anti-*M. leprae* human monoclonal antibodies is the first step in producing monoclonal human anti-idiotypic reagents which could potentially serve as vaccine replacements, as demonstrated in the case of African trypanosomiasis where administration of anti-Id in the absence of antigen protected against a subsequent challenge with pathogen (25). If monoclonal antibodies of this type are contemplated for human use, then the experience in the cancer field would suggest that human monoclonal antibodies could overcome the immunogenicity complication of mouse monoclonal antibodies (22).

In the present study, we used humoral response of LL patients to immortalize the human anti-*M. leprae* specificity repertoire in the form of human hybridomas. We have previously reported on the successful fusion of KR-4, a thioguanine-resistant, ouabain-resistant lymphoblastoid cell line, with a clone of Epstein-Barr virus (EBV)-transformed peripheral blood lymphocytes from tetanus toxoid-vac-

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cinated donors (18). Hybrids produced monoclonal, human, anti-tetanus toxoid antibodies. In this report, we fused KR-4 with EBV-transformed cell lines established from LL patients. Many hybridomas were derived which secreted monoclonal antibodies against various crude and purified *M. leprae* antigens.

MATERIALS AND METHODS

Patients. Lymphocytes from four treated LL patients were generously provided by W. F. Danby, Queen's University, Kingston, Ontario, Canada, and E. J. Shannon and R. B. Hastings, National Hansen's Disease Center, Carville, La.

Cell lines. EBV-transformed cells. Peripheral blood lymphocytes (10^6) from LL patient(s) were incubated for 2 h with 0.2 ml (2×10^7 transforming units) of mycoplasma-free supernatants from the EBV-producing cell line B95-8. Infected cells were then cultured at 37°C under 5% CO_2 in 24-well plates (Costar 3524) at a density of 1×10^6 per ml in RPMI-1640 medium containing 15% heat-inactivated fetal calf serum (FCS; GIBCO Laboratories), L-glutamine (2×10^{-3} M), gentamicin (50 μg/ml), and 2-mercaptoethanol (5×10^{-5} M). At 10 to 12 days after infection, cells were transferred to Falcon flasks and fed twice weekly with the above culture medium. The density of cells was kept at 0.5×10^6 to 1×10^6/ml.

KR-4. The human B lymphoblastoid cell line KR-4 was rendered resistant to ouabain and thioguanine as previously described (18). KR-4 cells were maintained at a density of 1×10^6 to 2×10^6/ml in culture medium containing 10^{-4} M ouabain (Sigma Chemical Co.) and 30 μg of 6-thioguanine per ml (Sigma).

Cellular hybridization. LL cells (10^7) and KR-4 cells (10^7) were washed four times in serum-free medium, mixed in 50-ml conical tubes (Falcon), and centrifuged at $150 \times g$ for 10 min at room temperature. The medium was removed completely and 0.5 ml of polyethylene glycol (molecular weight 4,000; Sigma) 45% (wt/vol) in RPMI was added to the cell pellet over 1 min. After 60 to 90 s of incubation with occasional mixing, 10 ml of warm (37°C) RPMI-1640 was added slowly to the cells over 10 min, and the cells were then incubated for 20 min at 37°C. This was followed by centrifugation for 4 min at $150 \times g$. The cells were then washed once and suspended in RPMI containing 20% FCS at 2×10^6 or 1×10^6/ml and seeded into 96-well flat-bottom microtiter plates (Linbro) at 0.1 ml per well. Irradiated (3,000 rads) spleen cells (0.5×10^6/well) and nonirradiated peritoneal exudate cells (5×10^3/well) from CDI mice (4 to 6 weeks old) (Jackson Laboratories) were used as feeder layers.

After 24 h, hypoxanthine aminopterin thymidine (HAT; Flow Laboratories) and ouabain (at 5×10^{-6} M final concentration) in RPMI–20% FCS were added to the cultures. The medium was removed 72 h after fusion, RPMI–20% FCS containing fresh HAT and ouabain was added, and the cells were fed every 4 to 5 days with the same selective medium for 2 weeks. The cells were then fed with HT medium (Flow Laboratories) containing only hypoxanthine and thymidine for 7 days. Finally, the feeding medium was changed to RPMI–20% FCS. Control cultures with parental cells (LL or KR-4) contained no surviving cells after 10 days in HAT and ouabain.

Cloning. Hybrid cells positive for anti-*M. leprae* antibody production were subcultured at 1 and 10 cells per well by limiting dilution in RPMI medium containing 20% FCS and 0.5×10^6 3,000-rad irradiated mouse spleen cells per well. Wells that were positive for antibody production were transferred to 24-well plates, expanded, and cloned at a density of 0.5 cells per well in the same culture medium and feeder layers.

Supernatants from clones were screened for anti-*M. leprae* antibody production, and positive clones were transferred to Falcon flasks and fed every 4 to 5 days in a medium containing 15% FCS. Supernatants from these clones were saved for further studies.

Organisms. *M. leprae* organisms were obtained from R. J. W. Rees (National Institutes for Medical Research, London, England) under contract to the World Health Organization and from Patrick Brennan (Colorado State University, Fort Collins) under contract to the National Institutes of Health. These organisms were isolated from infected armadillo tissue by World Health Organization protocol 1/79. Freeze-dried *M. tuberculosis* H37-10 was obtained from Connaught Laboratories, Montreal, Quebec, Canada, and M. bovis BCG (freeze dried) was from Institute Armand-Frappier, Laval, Quebec, Canada. The other mycobacteria used in this study were obtained lyophilized from the American Type Culture Collection, (Rockville, Md.) and grown on tryptic soy broth medium at 37°C.

Antigens. *M. leprae* and other mycobacteria were sonicated at 1 mg/ml in 0.9% NaCl on ice with a Braun sonicator at 80 W for 15 to 30 min (7) and centrifuged at $20,000 \times g$ for 15 min at 4°C (Beckman ultracentrifuge model L8). Samples of supernatants (MLS) were frozen at −70°C. Sodium dodecyl sulfate-soluble antigens (MSE) were prepared by solubilizing pellets from mycobacterial sonicates in SDS (Bio-Rad Laboratories) and 2% 2-mercaptoethanol (Sigma) by heating at 60°C for 20 min (5). Extractions with SDS were done immediately before each screening assay since storage in SDS and 2-mercaptoethanol was found to diminish antigenic activity.

A purified phenolic glycolipid (phen-GLIP-I) preparation from *M. leprae* (14) was generously provided by P. Brennan and judged to be highly pure from thin-layer chromatography analysis. The deacylated derivative of phenolic-glycolipid I (16) was generously provided by D. B. Young (Seattle Public Health Hospital, Seattle, Wash.). Crude lipid preparations from other mycobacteria were prepared in our laboratory according to the method of Reggiardo et al. (24). Briefly, freeze-dried mycobacteria were suspended in chloroform-methanol (2:1) and extracted at 50°C for 18 h in screw-cap tubes. Extracts were then centrifuged at $1,000 \times g$ for 30 to 40 min to obtain total soluble lipids that were dried under nitrogen and kept at 4°C. Further extraction was done immediately before each screening experiment with carbonate-bicarbonate buffer (pH 9.6) containing 1 mg of sodium deoxycholate per ml, followed by incubation at 56°C for 20 min.

ELISA. For enzyme-linked immunosorbent assay (ELISA), polystyrene microtiter wells (Dynatech Laboratories) were coated with 200 μl of 10-μg/ml equivalents of each antigen preparation (MLS or MSE) in 0.05 M carbonate-bicarbonate buffer, pH 9.6, and incubated overnight at 4°C in a humid chamber. The wells were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 with a standing time of 5 min between each wash. Bovine serum albumin (3%, 200 μl; fraction V, radioimmunoassay grade; Sigma) in PBS was added to each well and incubated for 4 to 5 h at 37°C to block nonspecific protein-binding sites in subsequent steps. The wells were washed and hybridoma supernatants were added and incubated overnight at 4°C. After the wells were washed three times, 200 μl of alkaline phosphatase-conjugated goat anti-human immunoglobulin

(Miles Laboratories) diluted in PBS-Tween 20 was added and incubated for a period of 2 h at room temperature. Plates were washed four times and the substrate solution, 1 mg of *p*-nitrophenyl phosphate per ml (Sigma) in diethanolamine buffer (pH 9.8) containing 0.001 M $MgCl_2 \cdot 6H_2O$ was added. After 30 to 45 min of incubation at room temperature, the reaction was stopped by using 50 μl of 3 N NaOH solution, and absorbances were read at 405 nm with a Titertek ELISA reader (Flow Laboratories).

For ELISA experiments with crude lipid antigens, polyvinyl chloride plates were coated overnight at 4°C with 50 μl of lipid extracted from the equivalent of 100 μg of mycobacteria per ml. Antigen was dissolved in carbonate-bicarbonate buffer, pH 9.6, containing 1 mg of sodium deoxycholate per ml. After wells were washed and blocked with 3% bovine serum albumin in PBS for 1 h at 37°C, culture supernatants were added and left for 1 to 2 h at 37°C and thereafter treated in the same manner as the ELISA experiments described above.

In all ELISA experiments, duplicate control wells consisted of (i) wells coated with buffer but lacking antigen and (ii) wells coated with antigen and LL serum at 1:500 added (positive controls) or 20% FCS in RPMI (negative controls).

Immunoglobulin assays. Polystyrene wells were coated with the $F(ab')_2$ fragment of rabbit anti-human immunoglobulin (Cappel Laboratories Inc.) at 1:1,000 in carbonate-bicarbonate buffer, pH 9.6, for 3 h at 37°C. Wells were washed, and after nonspecific binding sites were blocked with 1% bovine serum albumin-PBS for 90 min at 37°C, appropriate dilutions of purified IgG or IgM (Cedarlane Laboratories) or culture supernatants were added. After overnight incubation at 4°C, alkaline phosphatase-conjugated goat anti-human IgG (γ-chain specific) or anti-human IgM (μ-chain specific) (Cedarlane Laboratories) were added to the appropriate wells, and after a 2-h incubation and washing, *p*-nitrophenyl phosphate was added and absorbances were recorded as described above.

RESULTS

Frequency of fusion. Peripheral blood lymphocytes from LL patients were transformed with EBV and fused with the Thg^r Oua^r fusion partner KR-4. Cells were seeded at 2×10^5 per well, and the number of wells containing surviving hybrid cells after selection with HAT and ouabain was as high as 96 of 96. This corresponds to a fusion frequency of $>1.25 \times 10^{-5}$, and each well was therefore likely to contain more than one hybridoma. In subsequent experiments, fused cells were seeded at 10^5 per well. A comparison of the fusion frequencies between EBV-transformed cell lines and fresh peripheral blood lymphocytes from patient LL-1 is shown in Table 1. Hybrids between KR-4 and EBV-transformed cells yielded 91.8% of wells positive for growth, whereas hybrids between KR-4 and non-EBV-transformed cells from the same patients yielded only 2% of wells positive for growth. These results suggest that EBV-transformed lymphocytes are approximately 20 times more efficient at hybridizing to KR-4 than are nontransformed lymphocytes.

TABLE 1. Optimum conditions for hybridization between KR-4 cells and LL lymphocytes

No. of fusions[a]	Donor PBL[b]	No. of 96 well plates	No. of cells per well	Mean % wells positive for growth ± SD[c]	Fusion frequency ($\times 10^{-6}$)[d]
18	+EBV	18	2×10^5	91.8 ± 8.1	25.0
22	+EBV	44	1×10^5	47 ± 20.3	12.6
4	−EBV	4	2×10^5	2 ± 1.4	0.2

[a] 10^7 cells from one or more LL patients were fused with 10^7 KR-4 cells in the presence of 45% polyethylene glycol 4000.
[b] EBV-transformed (+EBV) or nontransformed (−EBV) lymphocytes were used for fusion. PBL, Peripheral blood lymphocytes.
[c] Wells were screened for growth positivity 12 to 15 days after fusion.
[d] Frequency was calculated from the mean fraction of negative wells by using the Poisson equation.

TABLE 2. Screening of hybrids between KR-4 and LL cell lines for anti-*M. leprae* antibody production

M. leprae antigen[a]	No. of hybrid wells screened for antibody[b]	No. of wells positive	% Positive
Sonic extract	1,760[c]	131	7.4
Soluble SDS extract	1,760[c]	240	13.6
Phenolic glycolipid	1,760[d]	9	5.1

[a] *M. leprae* antigen preparation used to coat wells.
[b] Polystyrene wells coated with soluble sonic extract or SDS-pellet extract or polyvinyl chloride wells coated with glycolipid antigen were reacted with 50 μl of hybrid supernatants. Positive controls contained LL serum pool diluted 1/500 and negative controls contained 15 to 20% FCS in RPMI. Optical density values (405 nm) higher than three times the mean of the absorbance of duplicate negative controls were considered positive. Hybridoma supernatants from all wells were split in half, and each half was screened against MLS or MSE antigen.
[c] Hybrid wells tested in five different experiments.
[d] Hybrid wells tested in two different experiments.

Screening for anti-*M. leprae* antibody production. Hybrids were screened for antibody by using three different extracts of *M. leprae*. In preliminary experiments, the ELISAs were validated by using LL sera, normal sera, and LL sera absorbed with *M. leprae* or other mycobacteria (T. Atlaw, M.Sc. thesis, Queen's University, Kingston, Ontario, Canada, 1983). Typical positive and negative control optical density values at 405 nm were, respectively, 0.80 and 0.01 for MLS, 1.0 and 0.01 for MSE, and 0.3 and 0.05 for phen-GLIP-I. Of a total of 1,760 growth-positive hybridoma wells that were screened, 131 hybrids (7.4%) produced anti-*M. leprae* sonic extract (MLS) antibodies and 240 hybrids (13.6%) produced antibodies against the SDS extract of insoluble cell walls (MSE). Of 176 wells, 9 (5.1%) produced antibodies against phenolic glycolipid from *M. leprae* (Table 2).

Cloning. Hybrids whose supernatants were positive in the ELISA were transferred to 24-well Costar plates, grown in medium containing 15% FCS, and then subcultured at 10 cells per well. Of 3,313 wells screened against all three *M. leprae* extracts, 7 to 11% were positive for antibody (Table

TABLE 3. Subculture and screening of hybridomas

M. leprae antigen	No. of hybrids subcultured[a]	No. of wells screened	No. of wells positive[b]	% Positive
Sonic extract	39	3,049	217	7.1
Soluble SDS extract	39	3,049	340	11.1
Phenolic glycolipid	5	264	23	8.7

[a] Hybrid cells producing anti-*M. leprae* antibody were subcultured at 10 cells per well in 20% FCS-RPMI in the presence of irradiated mouse spleen cells. When 2/3 confluence was reached, supernatants were removed and screened for anti-*M. leprae* antibody production by ELISA. Supernatants from all wells were split in half, and each half was screened against MLS or MSE antigen.
[b] Number of wells positive for anti-*M. leprae* antibody production.

TABLE 4. Screening of clones for anti-*M. leprae* antibody production

M. leprae antigen	Total no. of wells cloned[a]	No. of wells screened	No. of wells prositive	% Positive
Sonic extract	56	2,200	148	6.7
Soluble SDS extract	56	2,200	211	9.6
Phenolic glycolipid	6	168	21	12.5

[a] Cells were cloned at 0.5 cells per well by limiting dilution in the presence of irradiated mouse spleen cells as feeder layers. Supernatants from wells with growing clones were screened for antibody production. Supernatants were split in half, and each half was screened against MLS or MSE antigen.

3). Some of these cells were cloned by limiting dilution at 0.5 cells per well. A total of 2,200 clones were screened, and 148 (6.7%) were positive for anti-MLS and 211 (9.6%) were positive for anti-MSE antibody production (Table 4). Of 168 clones, 21 (12.5%) produced anti-phen-GLIP-I antibody.

Specificity. To investigate the specificity of the antibodies produced by the anti-*M. leprae* clones, crude extracts from four other mycobacteria (*M. tuberculosis*, *M. bovis* BCG, *M. smegmatis*, and *M. phlei*) were used in parallel screening experiments. These species have been shown in numerous studies to share many antigens with *M. leprae* (8–11, 28, 30). Results from representative clones are shown in Table 5. Of the total 122 clones screened against the five mycobacteria, 9 showed reactions restricted to *M. leprae* MLS, as illustrated by clones 5E and 8A, or to *M. leprae* MSE, as illustrated by clones 2C and 2D. Clone 3A and 4A reacted to both extracts of *M. leprae*, but were negative for all the other mycobacteria. At the other end of the spectrum, a non-specific clone, 83, produced antibodies to both extracts from every mycobacterium tested. Of a total of 58 clones screened, none showed specificity to *M. leprae* glycolipid antigen, since the seven representative clones shown in Table 6 also reacted with crude lipid extracts from at least one other species. Since we were comparing crude and purified antigens, negative reactions should be interpreted with caution. In both experiments (Tables 5 and 6), OD values of >3 times background were considered positive. Positive wells were usually ≥6 times background.

TABLE 6. Reactivity of clone supernatants to mycobacterial lipids[a]

Clones	Lipid extracts				
	M. leprae	*M. tuberculosis*	*M. bovis*	*M. smegmatis*	*M. phlei*
G-5D	+	+	+	+	+
G-5H	+	−	−	−	+
G-5F	+	+	−	−	−
G-10	+	+	+	+	+
G-4G	+	+	−	−	−
G-5B	+	+	+	−	+
G-6E	+	−	−	−	+

[a] Supernatants from clones reactive to the purified phen-GLIP-I antigen of *M. leprae* were screened for antibody production by ELISA, using 5 μg of lipid preparations from five different mycobacteria per well. This table shows representative experiments in which a total of 58 *M. leprae* reactive clones were screened.

Four of the hybridomas reacting with purified phen-GLIP-I also reacted with its deacylated derivative (Table 7). O.D. values obtained at high supernatant concentration were similar to those given by a murine hybridoma at low concentrations. The murine hybridoma has a known binding

TABLE 5. Screening of clone supernatants on panels of mycobacteria[a]

Clones	Mycobacterial antigen									
	Soluble sonic extract					SDS extract of pellet				
	M. leprae	*M. tuberculosis*	*M. bovis*	*M. smegmatis*	*M. phlei*	*M. leprae*	*M. tuberculosis*	*M. bovis*	*M. smegmatis*	*M. phlei*
2A	−	−	−	−	−	+	−	−	−	+
2C	−	−	−	−	−	+	−	−	−	−
2D	−	−	−	−	−	+	−	−	−	−
2H	−	−	−	−	−	+	−	−	+	+
3A	+	−	−	−	−	+	−	−	−	−
4A	+	−	−	−	−	+	−	−	−	−
4B	−	−	−	−	−	+	−	−	−	−
4H	−	−	−	−	−	+	−	−	−	+
5A	+	+	−	−	−	+	−	−	−	−
5E	+	−	−	−	−	−	−	−	−	−
6A	+	−	−	−	−	+	−	−	−	−
7A	+	+	−	−	−	−	−	−	−	−
8A	+	−	−	−	−	−	−	−	−	−
8C	−	−	−	−	−	+	−	−	−	+
9A	+	+	+	−	−	−	−	−	−	−
9D	+	+	−	−	−	+	−	−	−	+
9E	−	−	−	−	−	+	−	−	+	+
10D	+	+	+	−	−	−	−	−	−	−
10F	+	−	+	+	+	+	−	−	−	−
11A	+	+	−	−	−	−	−	−	−	−
12A	+	+	−	−	+	−	−	−	−	−
12C	−	−	−	−	−	+	−	−	−	+
12E	+	+	−	−	+	−	−	−	−	−
12H	+	−	+	+	+	−	−	−	−	−
83	+	+	+	+	+	+	+	+	+	+

[a] Supernatants from *M. leprae*-reactive clones were reacted with antigen-coated wells (10 μg/ml) of soluble sonic extract or SDS extract of pellet of five different mycobacteria including *M. leprae*. This data shows representative clones with specificity or no specificity to *M. leprae*. In this particular experiment, a total of 122 clones were tested.

TABLE 7. Reactivity of clones with deacylated phen-GLIP-I of *M. leprae*[a]

Source of antibody	Dilution	OD_{405}
Normal human serum (n = 4)[b]	1/20	0.153
BT[c] leprosy serum (n = 2)	1/20	0.291
LL serum (n = 4)	1/20	0.771
Murine monoclonal ascites (PG2B8F)[d]	1/2,000	1.408
Human hybridoma supernatant-clone G6	1/2	0.723
Human hybridoma supernatant-clone G-8	1/2	1.252
Human hybridoma supernatant-clone G-9	1/2	0.480
Human hybridoma supernatant-clone G-10	1/2	0.519
Medium-FCS control	1/2	0.050

[a] ELISA wells were coated with 5 μg of deacylated phen-GLIP-I per ml from *M. leprae*. Background OD_{405} (optical density at 405 nm) in nonantigen-coated wells were subtracted from values in antigen-coated wells.
[b] *n*, Number of donors used for serum pool.
[c] BT, Borderline tuberculoid.
[d] The IgM murine monoclonal PG2B8F (10) was generously provided by D.B. Young.

specificity for the trisaccharide region of *M. leprae* phen-GLIP-I (30).

Antibody class. IgM was the major immunoglobulin produced by three anti-*M. leprae* reactive clones (Table 8). Various amounts of IgM (0.1 to 10 μg/ml) and almost no IgG (0.03 μg/ml) were secreted.

Stability. Two randomly selected clones (83 and 40) produced anti-*M. leprae* antibodies in relatively high quantities for up to 10 weeks in culture, compared to clone 32 which showed a decline of antibody production beginning at week 4 in culture. All three clones studied were fast growing, with a doubling time of 20 to 24 h (Fig. 1). The reason for increasing antibody production in clones 83 and 40 is not clear, but it could be related to selection for fast-growing cells.

EBV-immortalized clones. EBV lines from three of the four LL patients were cloned at densities of 1 or 10 cells per well and screened for anti-*M. leprae* antibody production. This was done in an attempt to establish a line enriched for specific antibody-producing B-cells that could later be fused to produce hybrids with a much higher frequency of stable antibody production. A total of 10,000 clones of LL-EBV lines were screened, and only 42 (0.42%) were initially positive for anti-*M. leprae* antibody production against MLS or MSE. Upon retesting, only eight clones were still producing anti-*M. leprae* antibodies. These were grown in larger flasks, but the property of antibody production was lost gradually within 6 to 16 weeks (data not shown).

DISCUSSION

The results presented here demonstrate the feasibility of producing human monoclonal antibodies specific for *M. leprae* antigens by using peripheral blood lymphocytes from leprosy patients as donor cells. Ten of the hybridomas reacted with purified phen-GLIP-I, the trisaccharide of which is unique to *M. leprae* (11).

TABLE 8. Immunoglobulin isotypes produced by *M. leprae*-reactive clones

Clone	Selected with (Ag)[a]	Immunoglobulin (μg/ml)[b]	
		IgM	IgG
83	MSE/MLS	10	0.03
5B	MLS	0.8	0.02
G-10	Glycolipid	0.1	0.01

[a] *M. leprae* antigens used for screening in previous ELISA experiments.
[b] The amounts of immunoglobulin produced by supernatants from three clones was determined by ELISA with whole purified immunoglobulins as standards and μ or γ chain-specific, enzyme-conjugated, goat anti-human immunoglobulin.

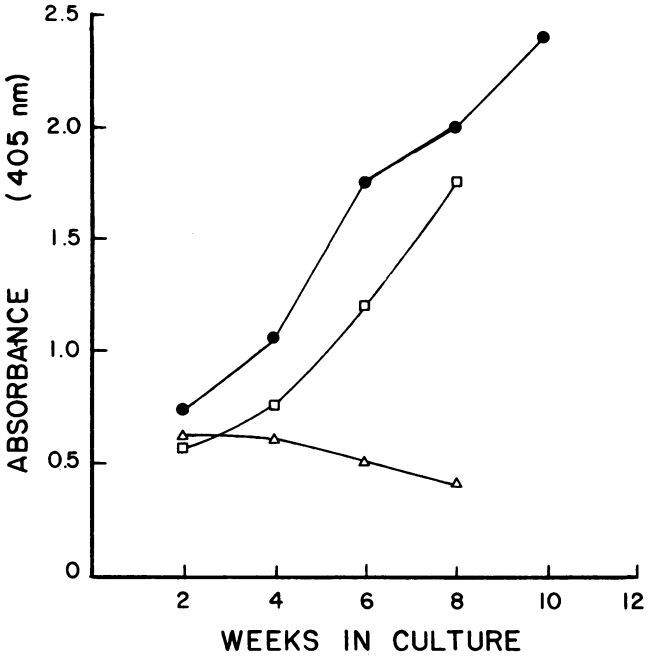

FIG. 1. Stability of antibody production by *M. leprae*-reactive clones. Supernatants from three anti-*M. leprae* antibody-producing clones were tested by ELISA once every 2 weeks for up to 10 weeks. Absorbance values were taken as quantitative indicators of anti-*M. leprae* antibody production. The antigens used were MSE for clone 83 (●), MLS for clone 40 (□), and MSE for clone 32 (△).

The use of EBV transformation alone, without hybridization, has yielded human monoclonal antibodies against some antigens (17, 31). However, only a few (42) anti-*M. leprae* clones were identified in the present study among 10,000 EBV-transformed clones from LL patients, and these lost their antibody-producing capacity within 6 weeks of culture. Loss of antibody production was previously observed at month 8 of culture in a clone of anti-tetanus toxoid-specific cells (17). In the present study, anti-*M. leprae* antibody production was rescued by fusion with KR-4, and hybridomas were stable beyond 12 months of continuous culture after two cloning cycles. The Oua^r Thg^r fusion partner KR-4 showed a 20-fold preferential hybridization to EBV-transformed donor lymphocytes compared to non-EBV-transformed lymphocytes from LL patients. Subculturing hybridomas at 10 cells per well after fusion, followed by a single cloning step for those anti-*M. leprae*-reactive wells, did not yield an increased percentage of anti-*M. leprae* reactive clones as expected, implying that hybridomas are initially unstable but stablize with time. EBV immortalization has several additional advantages since it allows one to repeat fusions on the same patient and produce and store large banks of EBV lines for eventual fusions. In addition, EBV is a polyclonal activator (20) and aids in the expansion of rare antigen-specific B cells before fusion.

The predominant class of antibody secreted by three anti-*M. leprae* hybridomas reactive with MLS, MSE, and phen-GLIP-I was IgM. Although antibodies to *M. leprae* glycolipid are usually IgM in leprosy patients (6), those to

antigens similar to MLS and MSE are IgG (D. Kozbor and J. C. Roder, Eur. J. Immunol., in press). In a previous report with the same EBV-hybridoma system, 20 of 21 anti-tetanus toxoid hybridomas were IgM, and only 1 of 22 was IgG (20). The donors were boosted with tetanus toxoid before fusion and had a predominant IgG serum response. It is possible, therefore, that EBV could bias hybridomas in this system toward the IgM class. Monoclonal IgM can be advantageous in some applications however, as in a murine malaria model, where it had the properties of an adjuvant during vaccination (12). Small amounts of monoclonal anti-malaria IgM potentiated both the primary and memory response to vaccination, as was revealed by enhanced survival after a lethal challenge infection. The human monoclonal antibodies described here could conceivably enhance the human response to the armadillo leprosy vaccine.

Most hybridomas produced were specific for antigens shared by a number of mycobacteria, as expected, since earlier studies with conventional antisera have documented extensive cross-reactions between different mycobacterial species (8–11, 26–28, 30). However, in a limited panel of mycobacteria, 9 clones of 122 reacted with undefined water-soluble or SDS-soluble antigens in *M. leprae* sonic extracts. Screening on a larger panel of mycobacterial species is necessary to establish the specificities involved. Several hybridomas reacted with a phen-GLIP-I extract of *M. leprae* and its deacylated derivative. Therefore the mycocerosic acids of phen-GLIP-I do not appear to be involved. The trisaccharide unit of these molecules is unique to *M. leprae* and binds only to serum antibodies from leprosy patients and not from normal subjects or patients infected with other mycobacteria (2, 3, 6, 23, 29). However, all of these clones reacted with crude lipid isolated from at least one other species of mycobacteria, and therefore, the determinants recognized are not unique to *M. leprae*. It is likely, therefore, that our hybridomas react with the genus-specific segment of this molecule, i.e., the phenolic phthiocerol core which is shared among the mycobacterial species, and further experiments have been designed to test this possibility. In contrast, reactivity of a murine hybridoma specific for phen-GLIP-I trisaccharide is restricted to *M. leprae* (30). Further experiments are in progress to determine whether binding of human monoclonal G8 to deacylated phen-GLIP-I can be competitively inhibited by a murine monoclonal PG2B8F against the trisaccharide portion of this antigen.

In summary, we have developed a system for producing human monoclonal antibodies against *M. leprae* antigens. The main advantage of these human reagents will be in the study of idiotype regulation in leprosy patients.

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