CMLS, Cell. mol. life sci. 53 (1997) 750–758
1420-682X/97/090750-09 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Augmentation of natural antiganglioside IgM antibodies in lower motor neuron disease (LMND) and role of CD5+ B cells

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Received 9 June 1997; received after revision 21 July 1997; accepted 28 July 1997

Abstract. IgM antibodies directed against neuronal gangliosides GM_1, GM_2, GD_{1a}, GD_{1b} and GT_{1b} occur in normal individuals and their level significantly decreases with age. Patients with lower motor neuron disease (LMND) produce high levels of these autoantibodies. AntiGM_1 IgM is selectively augmented. In these patients, the CD5+ (B1) and CD5− (B2) subsets of B cells are not distinct entities but range from those without detectable CD5 marker to those with high CD5+ expression. B1 B cells were sorted to homogeneity, but B2 B cell cannot be isolated to homogeneity because of the presence of B1 cells with low CD5 expression. In short term cultures both the subsets produced IgM antibodies, but the antibodies reacted better with desialylated GM_1 than with GM_1. Cycloheximide (Cx) (0.35 mM) largely blocked IgM synthesis of the B1 B cells but inhibition of the B2 B cells was incomplete, possibly due to shedding of cytophilic antibodies as well as to the presence of B1 phenotype with loss of CD5 expression. CD5+ B cells may be involved in the production of antiglycolipid IgM.

Key words. Gangliosides; antiganglioside IgM; CD5+ B cells; neuropathy; lower motor neuron disease (LMND).

Gangliosides (sialic acid-containing glycolipids) are immunosuppressive [1–8] and increase in the sera of cancer patients [8]. Antibodies are augmented to eliminate them from the circulation by immunizing patients with vaccines containing gangliosides [9–11]. As a consequence, a high level of antiganglioside IgM is correlated with improved survival of cancer patients [9, 10]. While repeated immunizations with purified gangliosides failed to induce antibodies [12], immunization with gangliosides mixed with foreign carrier proteins, erythrocytes and bacteria augmented antiganglioside IgM levels [13–15]. Mice immunized with ganglioside-containing cells or liposomes containing monophosphoryl lipid A (MPL) from *S. minnesota* augmented the production of antiganglioside IgM [16, 17].

Patients with lower motor neuron disease (LMND) produce high levels of antiganglioside IgM [21]. The persistence of very high levels of these IgM antibodies without downregulation or class switching of IgM to IgG, as one might expect in an immunization or an infection, and the factors responsible for their augmentation, are intriguing. The mechanism of enhancement of anti-ganglioside IgM is not clear although gangliosides may not require T-helper function for antigen presentation [18–21]. The following observations further document that the gangliosides are T-cell independent antigens: (1) gangliosides produce IgM antibodies in T-cell depleted mice (nude mice) [20]; (2) the reduc-

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Table 1. The antiglycolipid IgM antibody profile in the sera of normal individuals and patients with LMND.

Patient ID [1/1000]	Bckgrnd (*)	AsialoGM1 Gal-GalNAc-Gal-Glc-R	GM1 Gal-GalNAc-Gal-Glc-R | NeuAc	GM2 GalNAc-Gal-Glc-R | NeuAc	GD1b Gal-GalNAc-Gal-Glc-R | NeuAc-NeuAc	GT1b Gal-GalNAc-Gal-Glc-R | | NeuAc NeuAc-NeuAc	GT1a Gal-GalNAc-Gal-Glc-R | | NeuAc NeuAc
#1	0.017	0.091	0.474	0.223	0.184	0.114	0.15
#2	0.018	0.097	0.865	0.632	0.313	0.26	0.214
#3	0.017	0.367	1.346	0.302	0.183	0.167	0.259
#4	0.019	0.092	0.918	0.342	0.214	0.195	0.263
#5	0.029	0.074	0.649	0.212	0.17	0.13	0.219
#6	0.013	0.076	2.498	0.355	0.273	0.268	0.438
Normal [1/1000]	0.009	$0.044 + 0.03$	$0.018 + 0.017$	$0.038 + 0.02$	$0.096 + 0.03$	$0.057 + 0.04$	$0.055 + 0.03$

(*) Background values refer to ELISA absorbancy in wells without antigen. Normal values are represented as mean ± standard deviation of 10 normal individuals. 4 nmol/well of antigen used for all gangliosides to enable comparison of absorbancy values between reactivities with different gangliosides. All sera diluted 1/1000 in order to allow comparison of absorbancy values between the sera of different individuals.

tion in the level of these antibodies in patients after treatment with cyclophosphamide, an agent that acts directly on B cell mechanisms [21] and (3) the failure to reduce the same antibody titres with prednisone, a drug that acts mainly via T cell-mediated systems [21]. In addition, it is speculated that an 'unconventional' subset of B cells may be involved in the production of anti-ganglioside antibodies [22]. Peripheral blood lymphocytes of LMND patients were used to verify the above speculation after sorting of the major subsets of B cells.

In the peripheral blood, there are two major subsets of B cells distinguishable by cell surface markers. They are designated B1 and B2, with distinctive lineages and biological characteristics [23–27]. B1 cells are implicated in the production of natural IgM autoantibodies and in regulatory functions. In certain autoimmune diseases, the B1 cell subset has been expanded [28–32]. The surface marker available to distinguish B1 cells from B2 B cells is CD5, a 67 kDa protein which is also present on T cells. B1 cells are also called CD5+ B cells, although the designation is not entirely satisfactory because some of these cells may lack the CD5 marker [33, 34] and can be sorted as CD5− B2 cells. In this study, B1 and B2 B cells were isolated from the blood of neuropathy patients and maintained in short term cultures to find out which one of these B cell subsets is involved in synthesis and secretion of antiglycolipid IgM associated with LMND.

Material and methods

Human subjects. Normal donors were healthy adult volunteers with no evidence of infectious diseases, cancer, diabetics, atherosclerosis, rheumatoid arthritis or other autoimmune diseases at the time of blood collection. Those who had undergone surgery in the past three years or were scheduled for surgery were excluded. Sex, age, hair/eye/skin colour, ethnicity and major blood groups were recorded. As shown in table 1, six patients with lower motor neuron syndromes (LMND) were studied. Each patient had clinical and EMG examinations carried out by one of the authors (MCG). Sex and age of the neuropathic patients are indicated in table 2. The patients were selected for study because of elevated antibodies to GM1 or other glycolipid antigens. Motor nerve conduction block was found in cases 3 and 4 (multifocal motor neuropathy with conduction block) but not in cases 1, 2, 5 or 6 (axonal motor neuropathies). None had hyper-reflexia or bulbar symptoms, nor have these findings changed in 1 to 4 years of follow-up.

Flow cytometry of CD5+ and CD5− B lymphocytes. Venous blood samples (40 ml) from normal individuals and patients were collected in heparinized

Table 2. The relative proportions of CD5+/CD19+ (B1) and CD5−/CD19+ (B2) B lymphocytes in the peripheral blood of LMND patients and a normal individual.

Patient ID [sex/age]	Status	B lymphocytes [% of total lymphocytes]	CD5+/CD19+ [B1] [High density] % of total B cells	Total B1 cells % of total B cells	CD5−/CD19+ [B2] % of total B cells
#1 [M/57]	LMND	1685 [17%]	65 [4%]	625 [37%]	1060 [63%]
#2 [F/46]	LMND	910 [9%]	80 [9%]	380 [42%]	530 [58%]
#3 [F/36]	LMND	1045 [11%]	50 [5%]	580 [56%]	465 [44%]
#4 [M/41]	LMND	300 [5%]	4 [2%]	80 [27%]	220 [73%]
#5 [M/70]	LMND	145 [5%]	2 [1%]	35 [23%]	110 [75%]
#6 [F/47]	LMND	160 [7%]	10 [9%]	40 [25%]	120 [75%]
NORMAL	[n = 5]	895 ± 287 [9%]	14 ± 8 [2%]	154 ± 90 [17%]	727 ± 312 [81%]

These high values are not due to any contamination, such as cell debris or monocytes, since gating excludes them.

polypropylene 50 ml syringes. The peripheral blood lymphocytes (PBL) were removed by Ficoll-Hypaque (Sigma, St. Louis) centrifugation at 400 g at 20 °C for 30 min and washed (×2) in Hanks' balanced salt solution (HBSS) with 0.5% human AB heat-inactivated serum. Monocytes were depleted by transferring the cell suspension to culture flasks at 37 °C for 1 hr. The nonadherent cells were stained with mouse anti-human Leu 1 (CD5+) (IgG2a) coupled to Phycoerythrin (PE) and mouse anti-human Leu 12 (CD19) (IgG1) coupled to FITC (Becton-Dickinson Immunocytometry Systems, Mountain View, California), following the manufacturer's protocol (Monoclonal Antibodies: Source Book). Mouse IgG1-FITC and mouse IgG2a-PE were used as controls. For dual staining, both FITC- and PE-conjugated antibodies were used simultaneously. To confirm that staining of CD5+ cells is due to the CD5 molecule, we incubated cells with purified, unlabelled anti-human Leu 1 antibody prior to staining with PE-coupled anti-human Leu 1 (CD5+) monoclonal antibody.

A FACScan flow Cytometer (Becton Dickinson Immunocytometry Systems), equipped with a 15 mW 488 nm air/cooled argon-ion laser was used. Daily performance of the instrument was monitored by running 'calibrate beads' (Becton-Dickinson), using the autocomp software. This software program optimizes the forward scatter (FSC) and side scatter (SSC) setting to distinguish populations of lymphocytes from monocytes and granulocytes, based on their size and granularity, respectively, and automatically adjusts to fluorescence PNT compensation. The conventional lymphocyte gating excluded debris, red blood cells, lymphoblasts and monocytes [35]. 10,000 events/samples were collected. The purity of CD5+ B cells in the FACScan sorter is 100% (vide infra). The results were expressed as absolute numbers and as percentage of lymphocytes. For short term cultures, CD5+ and CD5− B cells were collected from the sorter under sterile conditions.

Short term in vitro maintenance of B cells. After sorting B cell fractions from patients #3, #4 and #6, the cells were maintained in 24-well plates in 3 ml of RPMI 1640 with 3.0% of inactivated human AB serum and antibiotics (Pen-Strep-Fungizone). The number of cells per well was adjusted to 5×10^4, The cells were incubated at 37 °C in 5% CO_2 for 21 to 25 days. On alternate days, 200 µl of fluid was replaced with an equal amount of fresh medium. Incubated culture medium without cells was used as a control. The media from experimental and control wells were analysed by ELISA. The absorbancy of the experimental media was corrected with the corresponding values of the control. The media in the control wells (without cells) did contain IgM antibodies from human AB serum used in the preparation. The absorbencies of the control ranged from 0.2 to 0.25 for anti-asialoGM_1 and from 0.25 to 0.3 for anti-GM_1. The absorbencies in different wells and at different points of time remained constant throughout the course of the experiment. For protein synthesis inhibition experiments, 0.35 mM (final concentration) cycloheximide (Cx)[1] was added at the initiation of cultures [36]. Cx inhibition studies were carried out on isolated B1 and B2 B cell fractions from two patients (#3 and #6).

Measurement of levels of antiglycolipid antibodies in ELISA. The level of serum and culture fluid antiganglioside antibodies was assessed by ELISA [37]. Serum IgM antibodies specific for gangliosides can be quantitated either by single point quantitation assay (SPQA) or by titrimetric assay (TA). In SPQA, serum is diluted once (1/100 or 1/1000) and tested against a known quantity of antigen in duplicate or triplicate and the values are expressed as absorbancy, whereas in TA the serum is diluted serially (1/100, 1/200, 1/400, 1/800 and so on), tested against the antigen in duplicate and the values are expressed as titres. Estimation of titres involves at least five-fold higher concentrations of each ganglioside antigen than is required for SPQA. Since

[1] At this concentration of Cx, the cells were viable for long periods. In this study, the viability of cells was indicated by intermittent secretion of antibodies by the lymphocytes.

this study involves a large collection of sera, the antigens are expensive and their availability in highly purified state is restricted; we therefore chose SPQA for quantitation of specific anti-ganglioside IgMs. Microtitre plates (Falcon-Probind, 3915) were coated with 100 µl of 4 nmole of glycolipid in ethanol, dried in vacuum as determined empirically [37]. The antibody binding was assessed using peroxidase-coupled goat anti-human IgM directed against µ chain (1/25,000) or anti-human IgG (1/25,000) (Jackson Immunoresearch Laboratories, Westgrove, PA). The absorbancy was measured at 490 nm in a UV Max kinetic microplate reader (Molecular Devices, USA). Background values refer to absorbancy obtained from well without antigen. The absorbancy of each anti-ganglioside antibody represents a mean of duplicate or triplicate values (as indicated in the legend for figures) obtained after correcting for the background. Serum obtained from a neuropathic patient with high absorbancy (>1.5) for GM_1, GD_{1b} and GT_{1b} served as a positive control. For the measurement of anti-glycolipid IgM in culture media, the medium incubated without cells served as a control. The final values were corrected for the background and control.

Results

Anti-glycolipid IgM in the sera of normal individuals. Analysis of sera (at 1/100 dilution) from healthy normal individuals of various ages revealed IgM but not IgG antibodies to common neuronal gangliosides, GM_1, GM_2, GD_{1a}, GD_{1b} and GT_{1b} (fig. 1). The level of IgM antibody significantly decreased with age. Furthermore, the levels of IgM antibodies as indicated by the absorbancy in SPQA differ markedly between different gangliosides, suggesting specificity of the IgM antibodies to different epitopes. The presence of IgM directed to different epitopes of the glycolipids suggests the existence of parent B cell clones secreting these antibodies in the circulation of normal individuals. We have earlier [38] isolated and immortalized a B cell clone from a cancer patient, secreting IgM specific for GM_2, to document the specificity of the binding.

Anti-glycolipid IgM in the sera of neuropathic patients. The results of the analysis of levels of serum antibody (1/1000 dilution) to different glycolipids of the neuropathic patients are presented in table 1. The absorbancy of serum antiglycolipid IgM antibodies of normal individuals at 1/1000 dilution is <0.1. Table 1 also shows the sugar moiety of the glycolipids, and the conformational entity which constitutes the antigenic determinant. IgM but not IgG against a variety of neuronal gangliosides was found to be elevated in the sera of these patients. The levels, particularly of anti-GM_1 IgM, are more than hundred-fold higher than that of normal sera. Patients #3, #4 and #6 showed very high levels of anti-GM_1 IgM (0.900). Patient #3 had the highest level for asialo-GM_1 IgM, although it was four-fold lower than that for anti-GM_1. Patient #2 showed high levels of anti-GM_2 IgM in addition to anti-GM_1 IgM. We have earlier documented that anti-GM_2 IgM secreted by a human B cell clone is monospecific [38].

Sorting of CD5+/CD19+ and CD5−/CD19+ B cells. B cell subsets from PBL were purified from three patients (#3, #4 and #6) with high anti-GM_1 levels. Figure 2A illustrates the range of cell size and granularity selected by gating, which exclude lymphoblasts and monocytes. The same gating was used for cell sorting, since inclusion of lymphoblasts and monocytes in short term cultures decreased the reproducibility of antibody values. The FACS analysis distinguishes CD5+ and CD5− B cell subsets. The FACS profile of lymphocytes obtained from three patients showed four stippled quadrants (fig. 2B–D). Each quadrant revealed a particular category of lymphocytes. The following four categories were identified from the four quadrants: (1) T cells: CD19−/CD5+; (2) CD19−/CD5− T cells; (3) CD19+/CD5− B2 B cells; and (4) CD19+/CD5+ B1 B cells. Unlike CD5+ CD19− T cells, CD5+ B cells showed a continuous rather than a bimodal distribution of CD5 antigen density (fig. 2B–D), suggesting that the density and expression of CD5+ on B cells in the blood may vary and that consequently the CD5− B cell population also includes CD5+ B cells with low or no expression of CD5. The flow cytometric analysis includes total number and percentage of cells in each quadrant. Table 2

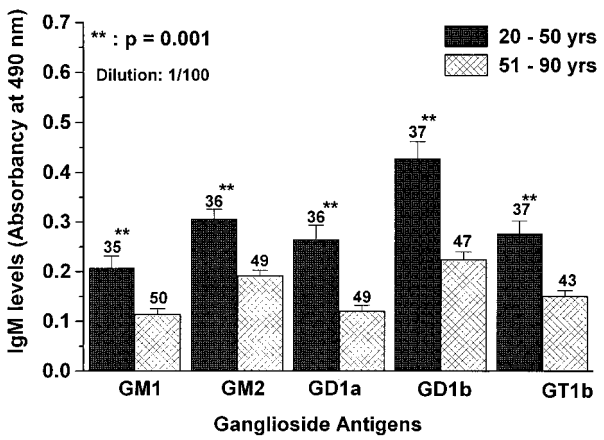

Figure 1. The levels of antiganglioside IgM antibodies directed against GM_1, GM_2, GD_{1a}, GD_{1b} and GT_{1b} in the sera of normal and healthy individuals decrease significantly with age (20–50 years to 51–90 years). The sera were diluted 1/100. Sample size for each age group is indicated above the error bars.

presents the percentage of different cell populations. The 'high density' CD5+ B cells, i.e. those with a CD5 density comparable to CD5+ T cells, constitute <10% of the total B cell population (table 2). The percentage of CD5+ B cells (20% to 56% of the total B cells) in patients is significantly higher ($p = 0.05$) than that in normal individuals (table 2). Pretreating cells with unlabeled anti-CD5 abolished the staining of both B and T cells with the labeled anti-CD5 reagent. The normal range of CD5+ B cells in healthy individuals (N in table 2 and in fig. 2E) is 10–25% of total B cells and is in line with other reports [11–14].

CD5+ B cells having a high density expression of the CD5 antigen were isolated (figs 2F and 2G) and incubated at room temperature for 5 h in sterile HBSS with 0.5% heat-inactivated human AB serum. Flow cytometry of these cells showed a small proportion (<5%) of CD5− B cells, suggesting that the B1 cells can lose the CD5 marker and sort with the CD5− fraction of B cells. The reverse transformation (CD5− B cells to CD5+) was not observed.

Secretion of anti-GM_1 antibodies in short term cultures of lymphocytes. Since there is great variation among B cell subpopulations from different individuals, we observed differences in both kinetics of antibody production and the level of antibodies produced in short term cultures. Figure 3A shows the changes in the level of antibodies produced in short term cultures of unsorted PBL. In contrast to serum, the IgM from the culture media reacted better to asialo-GM_1 ($p = 0.001$) than to GM_1. There is no correlation between serum levels and levels of in vitro produced antibodies among the patients included in the study. The peak of secretion was noted between days 8 and 10 and the level declined after the second week. The levels of anti-GM_1 and anti-asialoGM_1 IgM antibodies were assessed in the undiluted culture media of sorted B cell fractions obtained from three patients (#3, #4 and #6). In the culture media of CD5− B cells (from patient #4), the level of anti-asialoGM_1 IgM was highest on day 6, whereas in the media of CD5+ cells the peak was observed on day 8 (fig. 3B). The antibody level declined thereafter in both B cells.

Effect of cycloheximide (Cx) on antibody secretion. We assessed whether the antibodies found in the media are the result of synthesis by these cells or of the shedding of cytophilic antibodies which may have been absorbed prior to sorting, using Cx, an inhibitor of protein synthesis in the cell cultures (patients #3 and #6). Anti-GM_1 IgM was at a peak on day 8 in the wells containing CD5+ B1 cells and the peak disappeared in the presence of Cx (fig. 3C). Anti-asialoGM_1 IgM levels showed an increase on day 12 which is not observed (or which disappeared) in the presence of Cx (fig. 3C). The minor peaks were observed with or without Cx, suggesting that they may represent shed cytophilic antibodies. A similar increase in anti-asialoGM_1 was observed in cells obtained from patient #6. The media with CD5+ B cells showed significant anti-asialoGM_1 antibody levels between day 6 and 8 (ELISA O.D. 0.120 [day 6] and 0.044 [day 8], whereas the level declined markedly after treatment with Cx.

The culture media of CD5− B2 cells also contained anti-GM_1 IgM (fig. 3D) and anti-asialo-GM_1 IgM (fig. 3E). The level of anti-GM_1 IgM is reduced but not abolished in the presence of Cx. The peak of secretion of anti-asialo-GM_1 IgM was on day 10. However, this level of secretion occurred much earlier (on day 6) in wells with Cx (fig. 3E), suggesting that Cx may prompt release of preformed (cytophilic) antibody by CD5− B2 B cells. There is considerable difference betweeb two batches of B cells from the same individual with regard to anti-GM_1 antibody release into the culture medium in the presence of Cx. However, the time of secretion is relatively consistent. In CD5− B cells (from patient #6), low levels of anti-asialoGM_1 antibodies were detected on days 6 and 8 (0.029 and 0.052), which remained the same after Cx treatment.

Discussion

This study documents that the IgM antibodies directed against neuronal gangliosides are naturally occurring autoantibodies in normal individuals. The ELISA assay developed for assessing the fine specificity of antiganglioside antibodies [37] is useful for defining the fine specificity of the serum antiganglioside antibodies in normal individuals and LMND patients. The overall fine specificity profile of antiganglioside antibodies did not differ between young and old individuals, but there is a significant decrease in the level of all the IgMs in old age. There is a change in the level as well as a shift in specificity of antiganglioside IgM from normals to LMND patients (table 1). The most predominant antiganglioside IgM in the sera of LMND patients is anti-GM_1, suggesting that under certain pathological conditions such as LMND, the B cell clones secreting an antiganglioside antibody may either expand the specific clonal population or enhance secretion of the antibody. The augmentation of naturally occurring autoantibodies in the disease condition suggests that LMND may have the characteristics of an autoimmune disease. It is not clear at this point whether the pathological manifestations of the disease are the effect or cause of augmentation of antiganglioside IgM. However, an understanding of the factors promoting augmentation of antiganglioside IgM has tremendous therapeutic potential for treatment of patients with cancer (melanoma

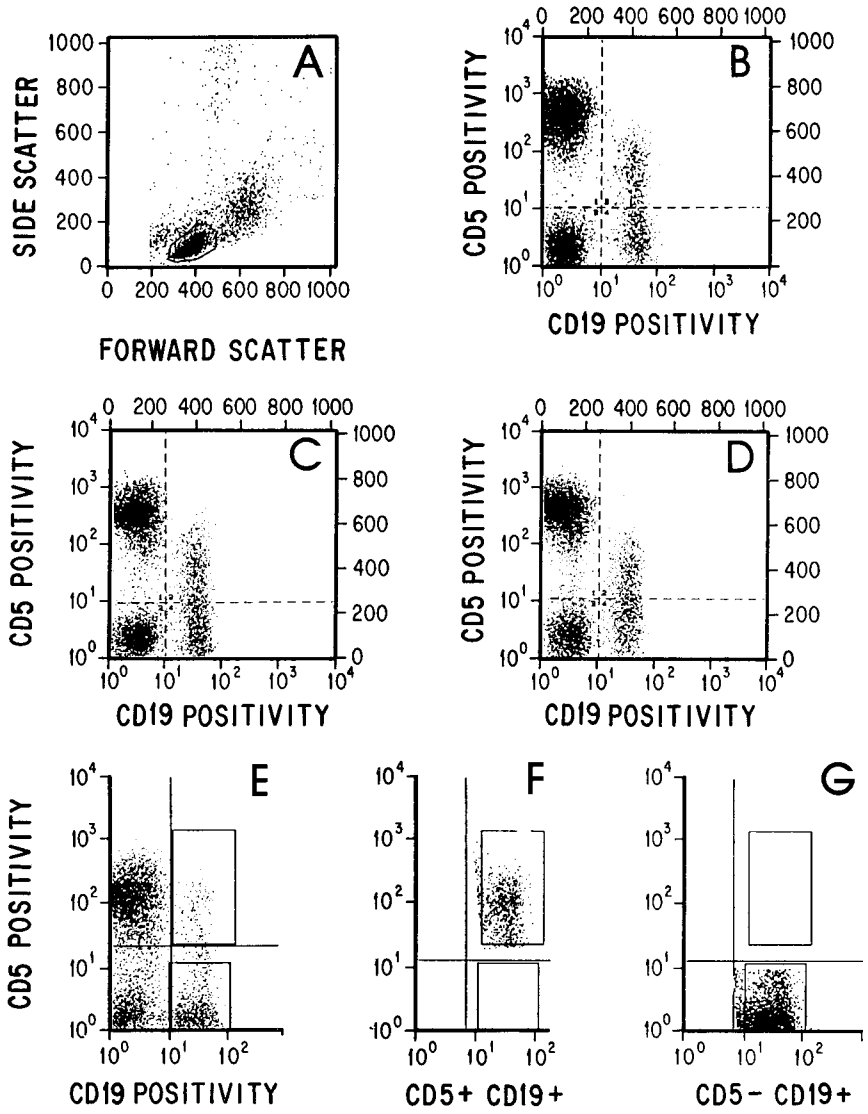

Figure 2. Flow cytometric profile of Ficoll-separated peripheral blood lymphocytes of neuropathy patients and normal individuals. Stained with fluorescein-conjugated anti-human Leu 12 (CD19+) (IgG1) and phycoerythrin-conjugated anti-human Leu 1 (CD5+) (IgG2a) monoclonal antibodies, and analysed on a dual laser FACScan flow Cytometer (Becton-Dickinson) equipped with a 15 mW 488 nm air-cooled argon ion laser. (*A*) A representative forward angle light scatter (FSC) and side angle light scatter (SSC) dot plot gate generated from PBL. The gate is restricted to distinct population of small lymphocytes (forward scatter 280–480). Large mononuclear cells comprising lymphoblasts and monocytes (FSC > 520) were excluded. Red blood cells and debris are also excluded by FSC threshold setting. (*B–D*) FACS profile of CD5+/CD5−/(B1) and CD19+/CD19− (B2) lymphocytes (total: 10,000 events) from LMND patients. (*E*) FACS profile of B cells obtained from PBL of normal individuals. The two distinct population in CD19− zones are T-cells with or without CD5 antigen. Note: CD5+ and CD5−CD19− cells are well separated. In contrast, CD19+ cells are not well separated for CD5 positivity. The intensity of CD5+ staining is much lower for CD19+ cells. There is a continuum of CD5+ antigen densities in different CD5+/CD19+ B cells. The overlapping zones among CD5+ B cells indicate that CD5+ expression on B cells vary markedly. (*F*) CD5+/CD19+ B cells. (*G*) CD5−/CD19+ B cells selected for short term cultures.

and neuroblastoma) expressing or shedding high levels of immunosuppressive gangliosides [1–10].

Expression of high levels of anti-asialoGM_1 IgM in culture media of B cells of these patients, in contrast to their respective serum, is noteworthy. We do not understand the significance of the shift in specificity of the IgM. Several possibilities can be suggested. The shift in the antibody may be imposed by the culture conditions in vitro. The culture medium might have triggered the B cells to synthesize and secrete anti-asialoGM_1 antibody. Alternatively, the low profile of anti-asialoGM_1 in the sera could be due to the formation of immune com-

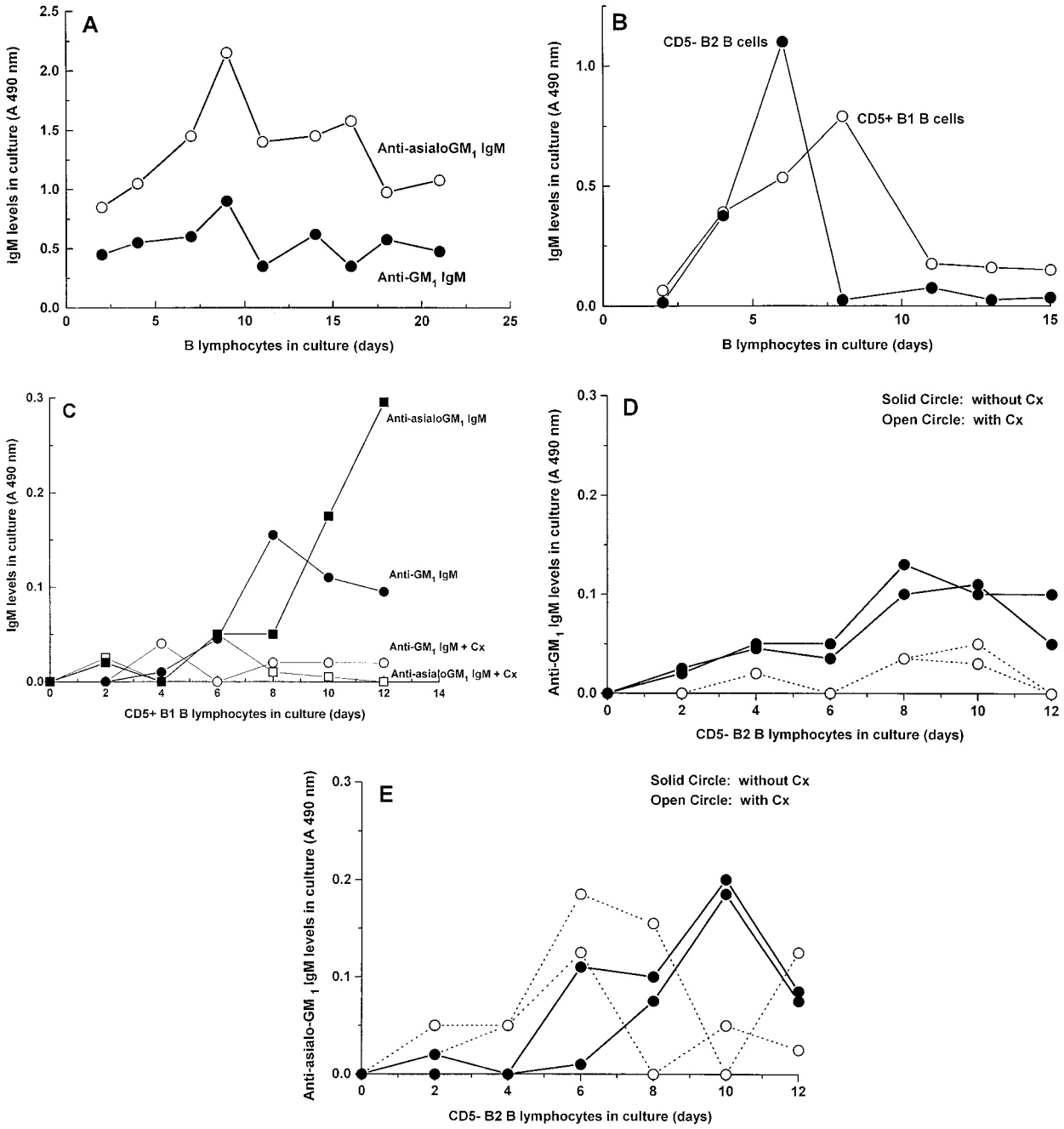

Figure 3. Production of antiglycolipid IgM (anti-asialoGM_1 and anti-GM_1) in short term cultures. (*A*) In unsorted lymphocytes (5.0×10^4) in culture. Source: patient #2. Cells were maintained in 250 µl of RPMI-1640 + 3.0% heat inactivated human AB serum + antibiotics in 96 well plates. Each value represents the mean absorbancy obtained from the media from eight wells. The media were tested without dilution and corrected for background and control (media without cells). (*B*) By purified CD5+ and CD5− B lymphocytes (5×10^4). Source: patient #4. Peak of secretion in CD5+ B cells occurred on day 8. A peak was observed for CD5− B cells on day 6. The level decreased drastically. The media were not diluted. The antibodies are not detectable after three weeks. (*C*, *D*, *E*) By purified CD5+ (B1) and CD5− (B2) B lymphocytes with or without cycloheximide (Cx). Number of cells per well: 5×10^4. Cx affected the viability of cells significantly (<15%). Source: patient #3. The antibody levels are expressed as mean of ELISA absorbancy of triplicate analyses. The experimental and control media were not diluted. Anti-GM_1 IgM shows a peak on day 8. A similar peak was observed for anti-asialo-GM_1 IgM. Cx-treated CD5+ B cells totally stopped secreting both anti-asialo-GM_1 and anti-GM_1 IgM antibodies on day 8 (*C*). Cx inhibition is partial in CD5− B cells. Two lines refer to two batches. Since CD5− cells are available in plenty after sorting, we had sufficient number of cells to run a second batch independent of batch 1. The responses of both batches were somewhat similar in their pattern of fluctuation. Anti-GM_1 IgM production is partially inhibited by Cx in wells containing CD5− B cells.

plexes with the antigen. Such a possibility is not surprising since a high level of circulating immune complexes is observed in neuropathic patients [40, 41].

Circulating B1 B cells are found to be elevated in the LMND patients, as in patients with autoimmune diseases such as rheumatoid arthritis, Sjogren's syndrome, type I diabetes and antiphospholipid syndrome [28–32]. However, we could not observe any correlation between antiganglioside IgM levels and the percentage of CD5+ B cells as in antiphospholipid syndromes [28], suggesting that a small number of cells might be exceptionally active in the production of antiganglioside IgM. While incomplete inhibition of antibody production by Cx may support the above contention, abrogation of antiglycolipid IgM production by CD5+ B1 cells by Cx suggests that these cells may be involved in the production of antiganglioside IgM. The role of CD5− B2 cells in the production of antiglycolipid IgM is inconclusive for the following reasons: (1) Cx inhibition of IgM production by the B2 cell fraction was partial; (2) the B2 cell population is not homogeneous and may contain B1 phenotype cells with low or no CD5 expression. In support of this, a 'sister' population of mouse peritoneal B cells which has B1 characteristics but lacks surface CD5 has been described [33] and shedding of CD5 marker by lymphocytes has been suggested [34]. However, our findings do not exclude the possibility of B2 B cells synthesizing anti-GM_1 IgM. In vitro synthesis of autoantibodies by cultured B cells is known in systemic lupus erythematosus (SLE) and other autoimmune disorders [39].

The role of viruses [42, 43] and bacteria such as *Camphylobacter jejuni* [44] in inducing antiganglioside IgM in these patients cannot be ruled out, since mice immunized with cells incorporating a bacterial derivative (MPL from *Salmonella minnesota* R595) augmented antiganglioside IgM levels [16, 17]. Bacterial products may interact with CD5+ B cells to augment the production of naturally occurring antiganglioside IgM.

In conclusion, the salient findings of this study are: (1) IgM antibodies against different species of gangliosides, commonly found in human neural and extraneural tissues, are found in the circulation of normal and healthy individuals; (2) these antiganglioside IgM antibodies may decline with age; (3) in patients with LMND, antiganglioside IgM antibodies are elevated, suggesting these antibodies could be the cause or effect of pathological conditions; (4) there is a specific increase in the level of anti-GM_1 IgM in LMND patients; (5) an unconventional subset of B cells may produce antiganglioside IgM antibodies. The characteristic feature of this subset of B cells is that it shares a T cell CD marker, CD5; (6) flow cytometric analysis shows CD5+ B cells are not a population distinct from the conventional CD19+/CD5− B cell population, because the range of expression of CD5 suggests possible loss of CD5 marker from these B cells during their ontogeny; (7) CD+ B cells secrete antiglycolipid IgM in short term cultures and their secretion is blocked by cycloheximide (Cx); (8) Cx partially inhibits the secretion of CD5− B cells, which could be due to the shedding of cytophilic antibodies and the presence of B1 B cells with loss of CD5 marker.

Acknowledgments. We are thankful to Dr Donald L. Morton for support and encouragement, and to Dr Jonathan Braun at UCLA for valuable suggestions. The experiments were done at Reed Neurological Research laboratory, UCLA. We wish to thank Mr David Bockstoce and Mrs Ingrid Schmidt for technical assistance in FACScan and cell sorter.

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