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Monoclonal antibody against a Burkitt lymphoma-associated antigen

(hybridoma/tumor antigen/Epstein–Barr virus)

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ABSTRACT **A monoclonal antibody, referred to as 38.13, was obtained by fusing murine myeloma cells with Lewis rat splenocytes sensitized with Daudi cells (human Burkitt lymphoma containing Epstein–Barr virus genome but lacking HLA-A, -B, and -C and β_2-microglobulin molecules at the cell surface). 38.13 antibody was demonstrated to be a rat IgM. By complement-dependent microcytotoxicity and indirect immunofluorescence assays, 38.13 antibody was shown to react specifically with cells derived from Burkitt tumors, including both Epstein–Barr virus genome-carrying and Epstein–Barr virus-negative Burkitt lymphoma. By contrast, Epstein–Barr virus-containing lymphoblastoid cell lines derived from normal B lymphocytes were not recognized by 38.13 antibody. Fresh malignant cells from patients affected with various lymphoproliferative disorders were negative, except 4/8 having abdominal Burkitt-like lymphomas. Normal lymphocytes from peripheral blood, spleen, lymph node, tonsil, and bone marrow and mitogen (phytohemagglutinin, pokeweed mitogen, and concanavalin A)-activated blasts were also negative. Thus, 38.13 antibody apparently recognized a Burkitt-associated antigen that is not related to Epstein–Barr virus. The pattern of reactivity of 38.13 antibody with various Burkitt lymphoma cells appeared quite heterogenous and some Burkitt cells were consistently negative. 38.13 antibody thus defines a subset of Burkitt lymphomas.**

Burkitt lymphoma (BL) is a B-cell proliferative disorder, of which there are two main forms, characterized by their geographical distribution and their relationship to the Epstein–Barr virus (EBV) (1). The African form was the first to be described (2) and is associated with EBV in most cases, as shown by the high anti-EBV antibody titers found in the sera of affected patients (3), the presence of integrated EBV genome in the DNA of the cells, and the expression of Epstein–Barr nuclear antigen (EBNA) in African BL cell nuclei (4, 5). In contrast, no clear association with EBV has been demonstrated in European and North American type BL; no EBNA and no viral DNA sequences can be found in most of these cases (6). It is of note that the histopathological features and the membrane marker patterns are similar in both types (7). Furthermore, a constant abnormality involving chromosome 8 (usually an 8 to 14 translocation) has been found in all BL cells (except BJAB), regardless of their geographical origin (8). On the other hand, when infected *in vitro* with EBV, normal B lymphocytes are polyclonally activated, and this process can lead to the establishment of permanent B-lymphoblastoid cell lines (9). Such immortalized B cells contain EBNA and DNA-integrated EBV genome (5). We report here the generation and characterization of a monoclonal antibody that reacts with malignant cell lines derived from both BL types but not with B cells from EBV-infected lymphoblastoid lines established from normal lymphocytes.

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MATERIALS AND METHODS

Cell Lines. The BL cell lines used were Daudi, BJAB, Raji, Ramos, Chev., P3HR1, Jijoye, BL9, Ly 81, Ly 91, Ly 46, Ly 47, Namalwa, and IARC/BL2. Most of these lines were provided by G. Lenoir from the Centre International de Recherches sur le Cancer (Lyon, France). The human lymphoblastoid cell lines used were Priess, Mich, T51, Rembl, MEN., LD, IARC 69, and IARC 74; they all originated from normal B lymphocytes. The T-cell lines used were Molt 4, JM, 1301, and Ke37 derived from T-cell leukemia. K562, obtained from a chronic myelogenous leukemia, was also studied.

All lines were cultivated in RPMI 1640 medium (GIBCO Bio-Cult, Scotland) supplemented with 10% heat-inactivated fetal calf serum.

Leukemia and Lymphoma Cells. The leukemia cells were obtained from peripheral blood of patients having various types of acute leukemia and chronic lymphocytic leukemia. The cells were separated by a Ficoll density gradient and frozen in culture medium/10% dimethyl sulfoxide or immediately tested. BL tumor and T-lymphoma cells were obtained frozen from A. Bernard (IGR, Villejuif), thawed, and tested in indirect immunofluorescence assays. Fresh malignant cells from two patients having Hodgkin disease and three patients having non-Burkitt B-cell lymphoma (two nodular types and one diffuse type) were isolated from lymph nodes or spleens and tested in indirect immunofluorescence assays.

Immunization. Immunization was performed using Daudi cells (10) derived from an African BL and chosen for the lack of expression on the cell membrane of HLA-A, -B, and -C antigens and β_2-microglobulin (11, 12). Daudi cells (2×10^7) in phosphate-buffered saline without adjuvant were injected intraperitonealy into three Lewis rats. This procedure was repeated 10 days later and the recipient animals were then intravenously boosted with 2×10^7 Daudi cells three days before hybridization.

Cell Hybridization and Selection of Hybrid Clones. Our cell fusion procedure was derived from the method of Köhler and Milstein (13) with the modifications described by Hämmerling *et al.* (14). The parental myeloma cell line was the murine non-immunoglobulin secreting MOPC 21 variant SP20/Ag14. Splenocytes (2×10^8) of the most effective antibody-responding rat were copelleted with 4×10^7 myeloma cells and fused by the addition of 40% polyethylene glycol 4000/phosphate-buffered saline. After fusion, aliquots of cell suspension in RPMI 1640 medium/10% fetal calf serum were placed into five tissue culture plates (Costar). Hypoxanthine/aminopterin/thymidine-h supplemented medium was added 24 hr later. The antibody-producing hybridoma selected was cloned twice by lim-

Abbreviations: BL, Burkitt lymphoma; EBV and EBNA, Epstein–Barr virus and nuclear antigen, respectively.

iting dilution in microtiter plates (Falcon) and injected intraperitoneally into pristane-primed *nude* mice to produce immune ascites.

Assays. All hybridoma supernatants were screened by complement-dependent lymphocytotoxicity tests using as targets a panel of lymphoid cell lines, including one EBV genome-carrying BL (Daudi), two EBV-negative BL (BJAB and Ramos), two EBV-positive lymphoblastoid lines (Priess and Mich), and one T-cell line (JM).

Cytotoxicity tests were performed in Terasaki plates: 1 μl of supernatant mixed with 1 μl of cell suspension (10^6/ml) was incubated for 30 min at 20°C, after which selected rabbit complement (5 μl, 1:8 dilution) was added for 30 min at 20°C. Then, 1 μl of trypan blue was added and the plates were checked with an inverted light microscope (Leitz, Diavert). After selection of BL-reactive supernatant, the specificity of the secreted immunoglobulin was further studied by both microcytotoxicity and indirect immunofluorescence assays.

The indirect immunofluorescence assays were carried out on fresh lymphoid cells in culture. Cells (10^6) were incubated for 45 min at 20°C with 50 μl of fluorescein-conjugated goat anti-rat immunoglobulin (Nordic). The results were unchanged when the incubation was at 4°C. All experiments included cells incubated with 50 μl of RPMI medium/10% fetal calf or normal rat serum (or purified rat IgM), before addition of goat anti-rat immunoglobulin conjugates as negative controls. After being washed three times, the cells were smeared on slides, air dried, and fixed in absolute ethanol for 10 min. Slides were examined with a Leitz Orthoplan microscope equipped with the proper combination of filters for fluorescein staining. Results are the mean of three independent experiments.

RESULTS

Generations of Monoclonal Antibodies. Among the 63 hybridoma colonies that had grown 3 weeks after the fusion, 16 supernatants, screened with a complement-dependent cytotoxicity test, exhibited various patterns of reactivity with BL target cells. One supernatant was selected for further study because of the high reactivity titer (up to 1:80) it showed with the BL-derived cells whereas it showed no activity against B- or T-lymphoblastoid cell lines. The corresponding hybridoma was cloned twice and the most active clone, referred to as 38.13, was selected. The 38.13 antibody was shown to belong to the IgM class by immunoelectrophoresis and Ouchterlony tests. The IgM fraction was purified on a Sepharose 4-B column (0.1 M Tris/0.5 M NaCl) and shown to contain the anti-BL activity.

Specificity of 38.13 Antibody. The 38.13 ascites had a lymphocytotoxicity reactivity titer of 1:40,000 on Daudi and Ramos cells. The specific reactivity of 38.13 ascites was tested with a panel of cell lines of various origins by both complement-dependent lymphocytotoxicity and indirect immunofluorescence assays.

Table 1 shows that all the positive target cells were of BL origin The lines reacting included EBV genome-carrying BL cells such as Daudi, P3HR1, Jijoye, Raji, BL9, Ly 81, Ly 91, and EBV-negative BL cells (BJAB, Ramos, Chev., IARC/BL2). However, various patterns of immunofluorescence reactivity were observed among BL cells. The most brightly and uniformly stained cells were from the EBV-producer Jijoye line and its subclone P3HR1. The BL cells Daudi (EBV^+), BJAB (EBV^-), Ramos (EBV^-), BL9 (EBV^+), LY 91 (EBV^+), IARC/BL2 (EBV^-) presented similar fluorescence reactivity, with 30–80% heavily labeled cells. On the other hand, a faint staining on 2–16% of cells was seen with Raji (EBV^+), Ly 81 (EBV^+), and Chev (EBV^-). Three BL lines (Ly 46, Ly 47, and Namalwa) were consistently negative. Namalwa was also found to be negative by an absorption test. None of the B-lymphoblastoid lines or T-cell lines showed any activity with 38.13 antibody.

Table 1. Reactivity of monoclonal antibody 38.13 with human lymphoid lines

Cell lines	EBV-genome integrated	Test: Immunofluorescence	Test: Cytotoxicity
African type BL			
P3HR1	+	100	100
Jijoye	+	100	100
Daudi	+	62	75
BJAB	+	46	50
BL9	+	43	50
Ly 91	+	35	50
Raji	+	16	25
Ly 81	+	14	25
Ly 46	+	0	0
Ly 47	+	0	0
Namalwa	+	0	0
European–North American type BL			
Ramos	−	71	100
IARC/BL2	−	82	75
Chev.	−	2	25
Normal lymphocytes			
Priess	+	0	0
Mich	+	0	0
T51	+	0	0
Rembl	+	0	0
Men.[1]	+	0	0
LD	+	0	0
IARC 69	+	0	0
IARC 74	+	0	0
T-cell leukemia			
Molt 4	−	0	0
JM	−	0	0
1301	−	0	0
Ke37	−	0	0
Chronic myelogenous leukemia			
K562	−	0	0

Immunofluorescence test results represent percent of cells labeled. Cytotoxicity test results represent approximate percent lysis.

To further establish that the antigen recognized by 38.13 antibody was BL specific, cells of various normal and malignant origins were tested.

Unfractionated peripheral blood lymphocytes, purified T or B cells, lymphocytes from normal spleens, tonsils, bone marrow and lymph nodes, and pokeweed mitogen-activated B blasts all appeared negative by indirect immunofluorescence assay. Thus, the antigen recognized by 38.13 has so far not been detected on normal lymphocytes.

Table 2 shows that malignant cells from various lymphoproliferative disorders were also negative with 38.13 The only exceptions were four cases of childhood abdominal B lymphosarcoma classified histologically and by surface labeling as BL. The majority of these neoplastic cells reacted with 38.13

Four human teratomas, one human melanoma, and one human neuroblastoma (15) were also tested and were consistently negative.

DISCUSSION

This study was concerned with the production and characterization of a monoclonal rat antibody, referred to as 38.13, that reacts with Burkitt lymphoma cells. The 38.13-producing hybridoma was obtained by fusion of Daudi cell-sensitized rat

Table 2. Reactivity of monoclonal antibody 38.13 with cells from various human lymphoproliferative disorders

Disease	Patients studied, no.	Positive results, no.
Chronic B-lymphoid leukemia	6	0
Acute lymphoid leukemia		
T	1	0
Non-T, non-B	2	0
Acute myeloid leukemia	2	0
Acute monoblastic leukemia	1	0
Hodgkin disease	2	0
T-cell lymphosarcoma	1	0
B-cell lymphosarcoma		
Nodular	2	0
Diffuse	1	0
B-cell lymphoma (Burkitt type)	8	4

splenocytes with the MOPC 21 variant SP20/Ag14 murine myeloma cells. The anti BL specificity of 38.13 antibody was established by screening its reactivity against various cell lines, normal lymphocytes and fresh malignant cells from patients having various leukemias and lymphomas by both complement dependent lymphocytotoxicity and indirect immunofluorescence assays. The only reactive cells included EBV-positive and EBV-negative BL and malignant cells from four of eight children affected with abdominal B-cell lymphoma histologically classified as Burkitt-like tumors. Other malignant lymphoid cells—including four T-leukemia lines; one chronic myelogenous leukemia line; and fresh cells from patients having T and non-B, non-T leukemias, T lymphoma, non-Burkitt B lymphoma (nodular and diffuse type), Hodgkin disease, acute myeloid, and acute monoblastic leukemia—were negative. Cultured malignant cells of various origins, such as four teratomas, one melanoma, and one neuroblastoma, were also unreactive with 38.13 antibody.

A normal counterpart of the membrane target antigen for 38.13 antibody has not been found so far on normal peripheral blood lymphocytes, bone marrow, tonsil, lymph node, or spleen cells from healthy individuals, nor on pokeweed mitogen- or phytohemagglutinin-activated blast cells.

38.13 Antibody thus appears to react with a Burkitt-associated antigen. Despite the presence of EBV in most of the BL cells studied, the corresponding antigen did not appear to be related to EBV; reacting BLs included both EBV genome carrying and EBV negative (Ramos, BJAB, Chev., and IARC/BL2). On the other hand, EBV-infected lymphoblastoid cell lines, in spite of containing EBNA and integrated EBV genome, were consistently negative. This was also true of the two EBV-producer marmoset cell lines B95-8 and M81. The reactivity of 38.13 antibody with Burkitt cells and not with lymphoblastoid cells was confirmed by fluorescence-activated cell sorter analysis (M. Lipinski, personal communication).

Recently, we tested simultaneously an EBV-negative BL line and a lymphoblastoid cell line obtained by EBV-infection of normal peripheral B cells (a gift from G. Lenoir) from the same patient. The BL cells were positive whereas the lymphoblastoid cells were negative.

The 38.13 antigen did not appear to be associated with a particular type of chromosomal lesion. Most of the BL cells reacting carry an 8 to 14 translocation, but in immunofluorescence assay, 38.13 stained others such as JBL 2 and Ly 91, which have a 2 to 8 translocation; IARC/BL2, which has an 8 to 22 translocation; Ly 65, which has a deletion in chromosome 8; and BJAB, which does not have any visible chromosomal damage. In double-labeling experiments, the 38.13 antigen was demonstrated to cap independently from surface immunoglobulin, and thus it is not associated with it at the BL cell membrane (unpublished results).

To characterize the BL antigen defined by 38.13 monoclonal antibody, attempts to immunoprecipitate a radiolabeled protein were performed with Ramos cells either surface labeled with ^{125}I- or metabolically labeled with [^{35}S]cystine or [^{35}S]methionine. After incubation with 38.13 antibody, Nonidet P-40-solubilized cell membranes were immunoprecipitated with a rabbit anti-rat immunoglobulin antiserum. Immunoprecipitates were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. No specific labeled protein could be identified. Furthermore, 38.13 antibody still reacted with Ramos cells after membrane treatment with various proteolytic enzymes including Pronase, trypsin, and α-chymotrypsin. These results suggested that the 38.13 target antigen was not a protein. In recent experiments, it was possible to inhibit the reactivity of 38.13 antibody by using the methanol/chloroform-extracted lipid fraction of Ramos cell membrane. This suggests that 38.13 antigen is a glycolipid (M. Lipinski, personal communication).

It is noteworthy that the pattern of reactivity of the monoclonal 38.13 antibody with various BL-derived cells appeared quite heterogenous. In most cases, BL cells were brightly stained in indirect immunofluorescence assays. The percentage of cells reacting ranged from 100% (P3HRl and Jijoye) to as low as 14% (Raji and Ly 81). One cell line (Chev.) was weakly positive and three others (Ly 46, Ly 47, and Namalwa) were consistently negative. Namalwa was also found to be negative by an absorption test. This heterogeneity was also observed with malignant cells from children having BL, which presented the same clinicopathological picture (Table 2). These data suggest either a variable expression of the target antigen during the cell cycle or the possibility of different maturational stages at which BL cells may be blocked. They also suggest the existence of heterogeneity among Burkitt lymphomas. However, it must be noted, that B lymphocytes at various maturational stages, including mitogen- or EBV-stimulated normal B cells and malignant B lymphocytes not of BL origin, all appeared negative.

Hybridoma-produced monoclonal antibodies may provide a unique tool for the investigation of tumor-associated antigens. The ability to obtain monoclonal antibodies reacting specifically with human tumor cells has been noted by Dippold *et al.* (16). By using a series of monoclonal antibodies, they defined various human melanoma-associated antigens, most of them being glycolipids. Ritz *et al.* (17) reported a monoclonal antibody to human acute lymphoblastic leukemia antigen, which they suggested to be leukemia specific. In BL, no such reagent has been described so far. A monoclonal antibody reacting with EBV-producing lines has been reported (18). This monoclonal antibody was clearly directed against an EBV-related structure; it did not react with EBV-negative cells and immunoprecipitated a 250,000-dalton glycoprotein of the EB virus.

Because it displays an apparent specificity for BL cells, the 38.13 monoclonal antibody could represent a valuable tool for diagnosis and specific immunotherapy of these lymphomas.

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