Apoptosis of Burkitt’s Lymphoma Cells Induced by Specific Interaction of Surface IgM With a Self-Antigen: Implications for Lymphomagenesis in Acquired Immunodeficiency Syndrome

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In a previous study, we described a cell line (BRG-P) derived from a woman with Burkitt’s lymphoma (BL) and acquired immunodeficiency syndrome that shared the same characteristic cytogenetic abnormalities as the patient’s malignant cells. This cell line contained subclones that displayed an isotype switch from IgM to IgA1 and an accumulation of point mutations in the V 

chains.\(^2\) The localization of replacement substitutions in sections. The antigen recognized by the BRG MoAb consisted of a single, minimally glycosylated polypeptide chain of 45 kD (p45). The BRG MoAb failed to react with a panel of human cell lines from different tissues, except for one cell line from a uterine cervical carcinoma. No reactivity was detected for a panel of exogenous antigens from various pathogens, including human immunodeficiency virus and self-antigens frequently recognized by polyspecific antibodies. Experiments were performed to investigate the functional consequences of the interaction of surface IgM with its specific ligand. Coculture of BRG-M cells with p45\(^+\), but not with p45\(^-\), breast cells caused apoptosis of BRG-M cells. The specificity of the interaction was shown by the observation that apoptosis was prevented by pretreatment of BRG-M cells with a monoclonal Fab\(^+\) fragment of rabbit IgG antibody to human \(\mu\) chains. Moreover, only BRG-M cells, but not other BL cells, underwent apoptosis after exposure to p45\(^+\) breast cells. The interaction between the CD40 molecule expressed by BRG-M cells and its specific ligand (CD40L) prevented p45-induced cell apoptosis. Because this interaction mimics that occurring in vivo between T and B cells during immune responses, our data suggest that various events contributed to the emergence of the BL, in this particular patient, including antigenic stimulation possibly assisted by T-cell help.

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B U R K I T T ’ S L Y M P H O M A (BL) is a neoplasia characterized by cytogenetic and molecular abnormalities consisting primarily of a specific chromosomal translocation that causes the juxtaposition of the c-myc proto-oncogene with one of the genes encoding the H or the L chains of Ig.\(^1\)\(^3\) The two types of BL, referred to as sporadic or endemic, differ in the prevalence of Epstein-Barr virus (EBV)-genome expression and in the topography of breakpoints of the chromosomal translocations.\(^4\)\(^5\) There is an increased incidence of BL in acquired immunodeficiency syndrome (AIDS).\(^6\)\(^8\) The majority of these BL cases are EBV-negative, although variations exist.

Although EBV is believed to play a major pathogenetic role in endemic BL by promoting B-cell proliferation,\(^9\)\(^10\) it is more difficult to determine the driving forces that contribute to the expansion of EBV-negative BL cells in the presence or absence of human immunodeficiency virus (HIV) infection. However, the excessive B-cell activation occurring in HIV-seropositive individuals suggests a potential role for stimulating antigens derived from a variety of pathogens as well as for self-antigens in this process. The hypothesis that antigenic stimulation may be involved is supported primarily by the observation that, in BL cells, point mutations, resulting in significant amino acid substitutions, can be detected in the gene segments encoding for the complementarity-determining regions (CDR) of the antibody H and L chains.\(^11\)\(^14\) The localization of replacement substitutions in the CDR, and not in the framework, is thought to occur during antigen-driven proliferation of B cells and to require T-cell assistance.\(^15\)\(^17\)

The availability of a continuous cell line from an AIDS patient (BRG) with an EBV-negative BL provided the opportunity to investigate the role of antigen stimulation in lymphomagenesis. The cell line (named BRG-P) expressed the same monoclonal \(\lambda\) chains and shared the same cytogenetic and molecular features as did the malignant B cells isolated ex vivo. Other unique features of BRG-P included an isotype switch that had occurred in vivo and had yielded IgM and IgA1 producing cell subclones in vitro and the presence of point mutations resulting in amino acid replacements in the CDRs of the H chain. Both events suggested that, in the course of maturation and lymphomagenesis in vivo, B cells were subjected to antigenic stimulation with the help of T cells.\(^11\)

The aims of the present study were (1) to identify the
antigen responsible for the stimulation of BRG cells and (2) to determine the effects of the interaction between this specific antigen and the membrane Ig of the malignant cells. The patient’s clinical course showed lymphomatous infiltration of the breast. Thus, we postulated that malignant B cells could react with an antigen expressed by breast tissue. We show here that the IgM produced by the lymphoma cells had specificity for an antigen of the breast ductal epithelium and that interaction between surface Ig and its specific antigen resulted in apoptosis of the malignant BRG cells in vitro. This apoptosis was prevented by exposure of BRG cells to CD40 ligand (CD40L), a molecular structure expressed by activated T cells and certain other cells that is important for their interaction with B cells.18,19 Our findings suggest a model for lymphomagenesis in which different events, including autoreactivity, antigen selection, and the help of CD40L-expressing cells, contribute to the expansion of the malignant clone.

MATERIALS AND METHODS

Description of the patient and derivation of lymphoma cell lines and of hybridomas. The case of patient BRG, a 30-year-old woman who was seropositive for HIV, was reported previously.11 The patient developed a mammary nodule that was diagnosed as a high-grade B-cell lymphoma. Peripheral blood (PB) smears and bone marrow biopsy at diagnosis showed the presence of a small number (2% to 5%) of Burkitt’s-type (L3) leukemic blasts. Surface marker analyses of PB mononuclear cells (PBMC) showed a small population of cells that coexpressed IgM, CD10, and CD38. The number of circulating neoplastic B cells increased during the course of the disease and the patient died of disseminated lymphoma. Chromosomal analyses resulted in a t(8;14) translocation.

A B-cell line (BRG-P) was derived spontaneously from the patient’s PBMC, as previously described.11 The majority of BRG-P cells expressed IgM and a minority were positive for surface IgA. Subclones that expressed only IgM or IgA1 and that shared the same c-myc and Ig H chain gene rearrangements as BRG-P cells were obtained by limiting dilution.

Hybridomas were obtained by fusing the cells of one IgM producing subclone (BRG-M) with those of the SHM D-33 heteromyeloma line.20,21 Hybridomas were selected in hypoxanthine, aminopterin, thymidine (HAT) medium (Seromed-Biochrom KG, Berlin, Germany) and ouabain (Sigma Aldrich, Milano, Italy) and cloned by limiting dilution. Supernatants of clones were screened for IgM production by an enzyme-linked immunoabsorbent assay (ELISA) and subsequently tested for their reactivity with various cell lines by immunofluorescence as described below. The supernatant of a single hybridoma (BRG-H) was selected for further testing. BRG-H cells shared the same H chain gene rearrangement as BRG-P parental cells, as shown by Ig VH gene fingerprinting analyses (kindly performed by Dr Mariella Dono, as detailed in Wakai et al”.

To purify IgM from the cell supernatants, BRG-H cells were cultured for 7 days in HyBM-ha medium (Promocell, Heidelberg, Germany) supplemented with SFM (Promocell). The supematants were concentrated in an ultrafiltration cell (Amicon, Co, Danvers, MA) and passed through a Sephacryl-S300 column (Pharmacia Biotech, Brussels, Belgium).

Immunofluorescence. The supernatant of BRG-H cells or its purified IgM fraction (from now on referred to as BRG monoclonal antibody [MoAb]) were used in indirect immunofluorescence tests. A mouse antihuman IgM (Becton Dickinson & Co, San Jose, CA) was used as a second step reagent, and a fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG1 antibody was used as a third-step reagent (Southern Biotechnology Associates, Birmingham, AL). In control tests, the supernatant of CLL 35.22 hybridoma was substituted for the BRG MoAb. This hybridoma was obtained by fusing IgM-producing chronic lymphocytic leukemia cells with cells of the HMMA heteromyeloma cell line, as described.22 All samples of stained cells were analyzed by flow cytometry (FACScan; Becton Dickinson). In selected experiments, immunofluorescence was performed on cells preincubated in RPMI-1640 medium (Seromed) containing trypsin (100 μg/mL) (Sigma) or promase (100 μg/mL; protease VI, Sigma) for 1 hour at 37°C.

An anti-CD40 MoAb was kindly donated by Dr S.M. Fu (University of Virginia, Charlottesville, VA) and an anti–Apo-I/Fas/CD95 MoAb was a generous gift of Dr P. Krammer (DKFZ, Heidelberg, Germany). Both MoAbs were used in indirect immunofluorescence as described.11

Reactivity of BRG MoAb with antigens from pathogens or self-antigens. The BRG MoAb was analyzed for its reactivity with double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), or rabbit γ-globulin (RGG) by ELISA, as described.11 Reactivity against EBV viral capsid antigen (VCA; Dupont-DeNemours, Bad Hamburg, Germany), rubeola virus (Clone Systems, Bologna, Italy), cytomegalovirus (CMV; Clone System), toxoplasma (Clone System), and chlamydia (Sorin Biomedica, Vercelli, Italy) was assayed by ELISA. Reactivity for antigens from HIV-1, HIV-2, human T-lymphotropic virus-1 (HTLV-1), and HTLV-2 was determined by ELISA (Sanofi Diagnostics, Pasteur SA, Marnes la Coquette, France) and Western blot assays. Binding to poliovirus type 1, 2, or 3 was analyzed using neutralization tests. Reactivity to self blood group cold agglutinins was tested by hemagglutination using group O umbilical cord or adult human erythrocytes.

Cell lines and cell cultures. The following human cell lines were used: MDA-MB-468, MDA-MB-231, MDA-MB-157, MCF-7, and EVSA-T (from breast adenocarcinomas); HELA and A431/6 from cervical carcinomas; NEVO from skin carcinoma; and SKMEL-28 from a melanoma. CALU1, CALU6, LC89, and A549 were derived from lung carcinoma; N592 from a lung small cell cancer; Y79 from a retinoblastoma; HT1080 from acetalbumin fibrosarcoma; and KS from spindle cell Kaposi sarcoma. All these cell lines were obtained from the American Type Culture Collection (ATCC; Rockville, MD), except for EVSA-T and LC89, which were raised as detailed in Paganuzzi et al.14 MCF-7 and EVSA T-cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Seromed, Berlin, Germany), whereas the others were cultured in RPMI-1640 medium. Media were supplemented with 10% fetal calf serum (FCS; Seromed), 1% glutamine, 1% Na pyruvate, and 2% penicillin-streptomycin. One BL cell line (LAM) was originated from an AIDS patient, as previously reported,23 whereas the BJAB BL cell line was obtained from ATCC.

MDA-MB-468 cells were fractionated according to the expression of the antigen recognized by BRG MoAb, using an immunorosetting method described previously.24 Briefly, cells were sensitized with BRG MoAb, followed by a mouse IgG1 antihuman IgM MoAb as a second-step reagent (Becton Dickinson). The cells were subsequently resorbed with ox erythrocytes coated with a purified goat antiamouse Ig antibody (Southern Biotechnology Associates) and rosettes were separated from nonresorbing cells by Ficoll-Hypaque density gradients.

Immunohistochemical staining. Seven samples of breast tumors were studied. Four of these were invasive ductal carcinomas, one was an intraductal carcinoma (micropapillary variant), and two were invasive lobular carcinomas. Cryostat sections were air-dried and fixed with cold acetone for 5 minutes. After rinsing with Tris-buffered saline (TBS), sections were incubated for 1 hour at 4°C with a goat antihuman Ig antiserum (Southern Biotechnology Associates) to block human Ig infiltrating the tissue. Rinsing with TBS was followed by overnight incubation at 4°C with BRG MoAb. After
another rinsing with TBS, sections were incubated for 45 minutes at 4°C with a 1:1 mixture of a mouse IgG1 antihuman IgM MoAb (Becton Dickinson; 1:10 in TBS) and of purified human IgG (1 mg/mL in TBS). This step was followed by routine processing for alkaline phosphatase antialkaline phosphatase (APAAP), ie, sections were incubated for 30 minutes at 4°C with a rabbit antimouse IgG (Dako, Glostrup, Denmark) followed by AAPAP for 30 minutes at 4°C. The alkaline phosphatase reaction was shown using a standard chromogen as the substrate (Naphthol AS-MX Phosphate and Fast Red TR salt; Sigma). Controls for the above-mentioned reactions included the incubation of sections with all of the reagents, either without the BRG MoAb or with a supernatant of the IgM CLL 35.22 hybridoma (see above).

Biochemical characterization of the antigen recognized by BRG MoAb. Cells (10 x 10^6) from MDA-MB-468 or MCF-7 cell lines were lysed using RIPA (Trion 1%, 150 mM NaCl, 100 mM L Tris, pH 8, 2 mM EDTA). Cell lysates were subsequently reacted with BRG MoAb (10 µg) or with control IgM (10 µg). These were monoclonal IgM purified from the sera of two patients with Waldenström macroglobulinemia.27 A goat antihuman (Southern Biotechnology Associates) and Sepharose-bound protein A (Pharmacia) were subsequently added. The immunoprecipitates were pelleted and resuspended in 50 mM Tris HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, and 0.01% bromophenol blue (with or without 4% 2-mercaptoethanol [2-ME]), sonicated, boiled, electrophoresed through 5% to 10% acrylamide gels, and electroblotted onto Hybond C extra nitrocellulose membranes (Amersham International, Amersham, Bucks, UK). The membrane was probed with purified BRG MoAb, followed by a goat IgG antihuman-IgM (Cappel Organon Teknika Corp, West Chester, PA) and by 125I-protein A (Amersham International). In control tests, purified monoclonal IgM were used instead of the BRG MoAb. The gels were exposed to Hyper-Film MP (Amersham International). The films were scanned with a Cannel CLC700 scanner (Cannon Inc, Costa Mesa, CA) and elaborated with an Adobe Photoshop 3.0 program (Adobe System Inc, Mountain View, CA).

Endogenous labeling was performed as previously described.28 Briefly, 10 x 10^6 cells were cultured with 20 µCi/ml 35S-methionine-cysteine (Tran 35S-label; ICN Pharmaceuticals Inc, Costa Mesa, CA) for 18 hours, harvested, lysed with Nonidet-P40 (NP40; Sigma), and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). In selected experiments, cells were labeled in the presence of tunicamycin (25 µg/ml; Sigma).

Apoptosis assays. BRG-M cells were resuspended at a concentration of 3 x 10^5/mL in RPMI 1640 supplemented with 10% FCS (RPMI-FCS) and cocultured with MDA-MB-468 at the optimal BRG-M/MDA-MB-468 cell ratio of 4:1. In parallel experiments, apoptosis was induced by exposing BRG-M cells to F(ab')2 fragments of rabbit IgG antibody to human µ chains [F(ab')2-µ-Ab] coupled to polyacrylamide beads (Bio-Rad Laboratories, Richmond, CA), as previously described.29 This reagent was used at a concentration of 10 µg/mL based on the results of preliminary titration experiments.

Apoptosis was measured by four different methods. First, permeabilized cells were stained with propidium iodide (PI; Sigma) and hypoploid cells were enumerated by flow cytometry.28 Second, DNA fragmentation was determined by electrophoresis according to a method previously described.29 Third, Giemsa-stained cytospin preparations were analyzed by light microscopy, and apoptotic cells were enumerated.29 Fourth, apoptotic cells were analyzed by electron microscopy. To this end, BRG-M cells were fixed with 2.5% glutaraldehyde (Polyscience, Warrington, PA) in 0.1 mol/L cacodylate buffer, pH 7.3, and post-fixed with 1% OsO4 (Polyscience) in the same buffer. After en bloc staining with 1% uranyl acetate and dehydration with ethanol and propylene oxide, samples were embedded in LX112 (Polyscience). Grey silver sections were stained with uranyl acetate and lead citrate, and observed with a Zeiss EM 10C electron microscope (Zeiss, Oberkochen, Germany).

Inhibition of apoptosis. To block the binding of surface IgM to the specific antigen, BRG-M cells were incubated with a monovalent F(ab')2 fragment of rabbit IgG antibody to human µ chains [F(ab')2-µ-Ab] before being exposed to MDA-MB-468 cells as above. F(ab')2 fragments were prepared by digesting rabbit IgG with 1% pepsin (Sigma). The resulting F(ab')2 fragments were purified on a Sephadex G150 column (Pharmacia) and subsequently reduced with 10 mM dithiothreitol (DTT) followed by alkylation with 30 mM L iodoacetamide (both from Sigma). Based upon the results of preliminary experiments, F(ab')2-µ-Ab was used at a concentration of 50 µg/mL.

In other experiments, apoptosis was inhibited with CD40L. The supernatant of mCD40L-CD8- α hybridoma cell line, kindly provided by Dr. P. Lane (Basel Institute for Immunology, Basel, Switzerland) was used as a source of CD40L.30 This hybridoma produces a fusion protein composed of CD40-L plus CD8-α. BRG-M cells at the concentration of 3 x 10^5 in RPMI-FCS were cocultured with MDA-MB-468 cell line or with F(ab')2-µ-Ab, with or without CD40L, at a molar ratio of 1:20. This dilution was selected based on the results of preliminary titration tests. Cells were analyzed at several time intervals for the presence of apoptosis.

RESULTS

Reactivity of IgM produced by BRG BL cells with breast cell lines. Immunofluorescence experiments were performed to test the reactivity of the IgM produced by BRG lymphoma cells with breast epithelial cell lines. To obtain soluble IgM antibody, a hybridoma (BRG-H) was made by fusing an IgM producing subclone of BRG-P cells (BRG-M) with the SHM-D33 heteromyeloma cell line. The IgM antibody produced, BRG MoAb, reacted strongly with cells from two breast cell lines (MDA-MB-468 and EVSA-T), but failed to stain cells of three others (MDA-MB-231, MDA-MB-157, and MCF-7). The IgM containing supernatants of a control hybridoma (CLL35-22) consistently failed to react with cells of any of the above cell lines (Fig 1). The reactivity of the BRG MoAb was abrogated by pretreatment of MDA-MB-468 cells with pronase or trypsin, indicating that the surface antigen recognized by BRG MoAb was a protein (Fig 1).

BRG MoAb failed consistently to react with cells of four human lung carcinoma (CALU1, CALU6, LC89, and A549), one small-cell lung cancer (N592), one skin carcinoma (NEVO), one retinoblastoma (Y79), one melanoma (SKMEL28), one fibrosarcoma (HT1080), and one spindle cell Kaposi sarcoma (KS). A weak fluorescence staining with a small, but significant shift of the flow cytometry profile was observed with cells from one uterine cervical carcinoma (A4316); another uterine cervical carcinoma cell line (HeLa) was negative (data not shown).

ELISA tests were also performed to determine the possible reactivity of BRG MoAb with a panel of self-antigens and exogenous antigens of common pathogens. These included dsDNA; ssDNA; RGG; and recombinant proteins of HIV-1, HIV-2, HTLV-1, HTLV-2, EBV, rubella virus, CMV, toxoplasma, and chlamydia. The results of all of these tests were consistently negative. Reactivity with HIV-1 and HIV-2 antigens, also tested by Western blotting, was negative.
Molecular characterization of the antigen detected by BRG MoAb. Initial attempts to characterize the molecule(s) in cell lysates reactive with BRG MoAb by standard Western blotting proved unsuccessful. To overcome these difficulties, the antigen(s) reactive with BRG MoAb were first immunoprecipitated from cell lysates and subsequently analyzed by immunoblotting on 10% acrylamide gels. A band of an apparent molecular mass of 45 kD was detected with this method in cell lysates of MDA-MB-468 cells. This band was present both in the gels run under reducing (data not shown) as well as under nonreducing conditions (Fig 2A). This 45-kD band was never detected in the immunoprecipitates of MCF-7 cell lysates (Fig 2A), thus confirming the immunofluorescence data. Several bands in the 50- to 60-kD region were observed in the immunoprecipitates of both MDA-MB-468 and MCF-7 cell lysates obtained with both BRG MoAb and with the control monoclonal IgM, possibly indicating nonspecific binding of human IgM to certain cellular structures (Fig 2A). The immunoprecipitates of cell lysates obtained with BRG MoAb were analyzed by immunoblotting using 5% gels. These analyses consistently failed to show molecules of a molecular mass greater than the 45-kD band described above. Thus, the 45-kD band was the only antigen recognized specifically by BRG MoAb.

In another set of experiments, MDA-MB-468 cells were cultured for 18 hours in the presence of a mixture of ^35^S-labeled methionine and cysteine. Cell lysates were subsequently immunoprecipitated with BRG MoAb or with two control human monoclonal IgM and analyzed by SDS-PAGE. Three or four bands were consistently observed in the 50- to 60-kD region in all of the immunoprecipitates, irrespective of the IgM reagent used for immunoprecipitation. However, a band corresponding to a molecular mass of 45 kD was detected only in the lysates immunoprecipitated with BRG MoAb, confirming its specificity for this

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Fig 1. Reactivity of BRG MoAb with certain epithelial breast cell lines. (A) IgM purified from the supernatant of BRG-H cell line (BRG MoAb) was used to stain the cells of MDA-MB-468 and MCF-7 epithelial breast cell lines (●) by indirect immunofluorescence. The negative control was performed using IgM from CLL 35.22 hybridoma (○). (B) Cells of MDA-MB-468 cell line were stained with BRG MoAb after trypsin or pronase treatment (□) or no treatment (■). Stained cells were analyzed by flow cytometry.

Fig 2. Molecular characterization of the antigen detected by BRG MoAb. (A) Analysis of immunoprecipitates obtained from MDA-MB-468 or MCF-7 cell lines with BRG MoAb or control human IgM as indicated. Immunoprecipitates were electrophoresed on 10% SDS-PAGE under nonreducing conditions. The gels were electroblotted and probed with purified BRG MoAb, followed by a goat IgG anti-human IgM antibody and by ^125^I protein A. The gels were exposed to Hyper-Film MP and scanned with a Cannon scanner. (B) SDS-PAGE analysis of the immunoprecipitates obtained from cell lysates of MDA-MB-468 cells endogenously labeled with ^35^S methionine-cysteine. The cells were labeled for 18 hours, lysed, and immunoprecipitated with two different control human IgM or purified BRG MoAb as indicated. The immunoprecipitates were electrophoresed on a 10% gel under nonreducing conditions. Autoradiograms of the gels were obtained on Hyper-Film MP and photographed.
molecular structure (Fig 2B). The mobility of the specific band did not change when SDS-PAGE was run under reducing conditions (data not shown), thus indicating that the protein antigen detected by BRG MoAb consisted of a single polypeptide chain. Moreover, the SDS-PAGE mobility of the 45-kD band did not change when cells were endogenously labeled in the presence of tunicamycin, which inhibits N-linked glycosylation. This indicates that the 45-kD protein was minimally N-glycosylated. Because of these findings and for the sake of brevity, from this point the antigen detected by BRG MoAb will be referred to as p45.

Reactivity of BRG MoAb with breast tissue. We next tested the capacity of BRG MoAb to stain tissue sections of breast carcinomas (Fig 3). A moderate to strong reactivity of the BRG MoAb was detected in the cells of alveolar portions of the malignant ductal epithelial cells. This notion was reinforced by observation of apparently normal alveolar portions of the ducts of both ductal (Fig 3A and C) and lobular carcinomas, whereas alveolar cells in invasive lobular carcinomas were consistently negative (data not shown). The reactivity of ductal cells in sections of invasive lobular carcinomas also indicated that p45 was expressed in normal as well as in malignant ductal epithelial cells. This notion was reinforced by observation of apparently normal alveolar portions of the gland that were present in the periphery of invasive ductal cancers. Here, the ducts were stained by BRG MoAb, whereas the alveolar portions of the gland displayed a negative or negligible reaction (Fig 3E and F).

Coculture with MDA-MB-468 cells causes apoptosis of BRG-M cells. Next, we investigated the effects of the interaction between surface IgM and its specific ligand (p45) on BRG-M cells. Because it has been reported that ligation of surface IgM by anti-μ antibodies causes apoptosis of BL cells in vitro, it was conceivable that interaction of surface IgM with p45 could cause a similar effect in the BRG-M cells. This hypothesis was explored in experiments in which BRG-M cells were cocultured with either MDA-MB-468 cells that expressed p45 (MDA-p45⁺ cells) or MDA-MB-468 cells that did not (MDA-p45⁻ cells). MDA-p45⁺ cells were obtained by removing p45⁺ cells from the parental MDA-MB-468 cell line. Removal of p45⁺ cells, which represented the majority of the cells in the suspensions (see Fig 1), yielded an almost pure population of p45⁻ cells. Flow cytometry analyses showed that these cells maintained the p45⁺ phenotype for 3 to 4 days after the separation procedure (data not shown).

BRG-M cells were cocultured with MDA p45⁺ or MDA p45⁻ cells and analyzed for apoptosis. Because both MDA p45⁺ and MDA p45⁻ cells were tightly adherent to the plastic surfaces, whereas BRG-M cells remained in suspension, it was possible to obtain, at the end of the culture period, cells that were composed predominantly of BRG-M cells, thus facilitating the evaluation of their apoptotic process. BRG-M cells were first stained with PI and analyzed by flow cytometry. Fifty percent to seventy percent of the BRG-M cells cocultured with MDA-p45⁺ cells underwent apoptosis (Fig 4A). These values were observed after 24 hours in culture and remained virtually unchanged for the next 72 hours. In contrast, only rare BRG-M cells cocultured with MDA p45⁻ cells were apoptotic. These findings were confirmed by DNA electrophoresis and by morphologic analyses, both at the light and at the electron microscopy level. DNA laddering and nuclear fragmentation, characteristic of apoptosis, were observed only in BRG-M cells cocultured with MDA-p45⁺ cells for 24 hours (Fig 4B and C). Electron microscopy analysis showed typical ultrastructural features of apoptotic cells in the BRG-M exposed to MDA-p45⁺ cells (Fig 4D). Notably, MDA p45⁻ cells did not undergo apoptosis irrespective of whether they were cultured in the presence or in the absence of BRG-M cells. This was determined by morphologic as well as by PI staining analyses on adherent MDA-p45⁻ cells recovered from the culture plates (data not shown).

Additional experiments confirmed that both the p45 molecules on the surface of target cells and the specific IgM on the surface of BRG-M cells were necessary to induce apoptosis. Thus, when BRG-M cells were cocultured with cells from the p45⁻ MDA-MB-157, MDA-MB-321, and FCMI7 cell lines, PI-stained and analyzed by flow cytometry, no apoptosis of BRG-M cells was observed above the already low background values. In contrast, cells of the p45⁺ EVSA-T breast cell line induced apoptosis of BRG-M cells (data not shown). Finally, MDA-p45⁺ cells consistently failed to cause apoptosis of cells from two other BL cell lines (LAM and BJAB).

Apoptosis of BRG-M cells is prevented by F(ab')₂-αμ-Ab. In subsequent experiments, BRG-M cells were exposed to a F(ab')₂-αμ-Ab at 4°C for 1 hour before coculture with MDA-p45⁺ cells. Cells were cocultured for 24 hours at 37°C before PI-staining and flow cytometry analysis of recovered BRG-M cells. Unlike the divalent F(ab')₂-αμ-Ab that induced apoptosis of the majority of BRG-M cells (Fig 5), F(ab')₂-αμ-Ab failed to induce apoptosis of BRG-M cells (Fig 5). This is consistent with the inability of this reagent to cross-link the cell surface IgM. However, the presence of F(ab')₂-αμ-Ab inhibited substantially the apoptosis induced by MDA-p45⁺ cells. Therefore, it appears that F(ab')₂-αμ-Ab failed to cause apoptosis of BRG-M cells induced by p45 molecules, also prevented the delivery of the apoptotic signal.

BRG-M cells are rescued from apoptosis by CD40L. Upon binding to its complementary structure (CD40) on the B-cell surface, CD40L prevents apoptosis of certain B-cell subsets such as germinal center cells. In these experiments, we tested whether apoptosis of BRG-M cells induced by exposure to MDA-p45⁺ cells could be inhibited by CD40L. Immunofluorescent studies showed that BRG-M cells expressed abundant surface CD40, as is typical for BL cells (data not shown and Holder et al¹⁰). BRG-M cells were exposed to MDA-p45⁺ cells in the presence or absence of CD40L. Cells were cultured for 24 hours, harvested, PI-stained, and analyzed for apoptosis by flow cytometry (Fig 6). Whereas exposure to MDA-p45⁺ cells caused apoptosis of a large fraction of cells (50% to 70%), the presence of CD40L resulted in almost complete inhibition of apoptosis. CD40L also inhibited apoptosis of BRG-M cells induced by F(ab')₂-αμ-Ab (Fig 6).

DISCUSSION

This study shows that the malignant B cells from a patient with BL and AIDS reacted specifically through their surface...
Fig 3. Staining of breast tumor sections with BRG MoAb or control CLL35.22 monoclonal IgM. (A and B) A ductal carcinoma invading the breast skin is strongly stained by BRG MoAb (A) and not by control CLL35.22 MoAb (B). Note that keratinocytes surrounding the tumor infiltrate are negative. (C and D) A neoplastic duct with intraductal papilliferous growth stains with the BRG MoAb (C) and not with control CLL35.22 MoAb (D). Alveoli adjacent to the duct are unstained (arrows). (E and F) An apparently normal mammary lobule in the periphery of a ductal carcinoma; ducts stain with the BRG MoAb (E) and not with control CLL35.22 MoAb (F). (Original magnification × 250.)
SELF-ANTIGEN-INDUCED APOPTOSIS OF BL CELLS

IgM receptor with a self-antigen (p45) expressed on human breast ductal cells. This interaction caused apoptosis of malignant BL cells that could be prevented by exposure to CD40L.

A monoclonal IgM (BRG MoAb), produced by an hybridoma obtained from BRG-M cells, reacted specifically with p45 antigen and failed consistently to bind antigens from several pathogens, including HIV. Moreover, the BRG MoAb did not react with molecules such as RGG, dsDNA, and ssDNA that represent common targets of polyreactive antibodies, thus indicating that the BRG MoAb had a restricted range of specificity. Finally, the BRG MoAb failed to react with cells from a panel of human cell lines mainly of epithelial origin, with the exception of those from human cervical carcinomas.

Immunohistochemical observations of breast sections showed that p45 was expressed selectively by ductal cells, both malignant and normal. Although normal tissue samples were not available, this conclusion was derived from analyses of normal alveolar tissue that surrounded ductal tumors and of uninvolved ductal epithelium present in lobular carcinomas. Because histopathologic analyses indicated that the patient did not have breast cancer, the native p45 autoantigen expressed by her normal breast cells was likely to be the target for the BL cells. This would have, in turn, favored

Apoptosis of BRG-M cells

in the presence of:

- \( F(ab')_2\mu\text{-Ab} \)
- \( F(ab')_2\mu\text{-Ab} \)
- MDA p45 cells
- MDA p45 cells + \( F(ab')_2\mu\text{-Ab} \)

Fig 5. Inhibition of apoptosis of BRG-M cells by a monovalent \( F(ab')_2\mu\text{-Ab} \). BRG-M cells were incubated with \( F(ab')_2\mu\text{-Ab} \) (10 \( \mu\text{g/mL} \)) for 1 hour at 4\(^\circ\)C and placed in culture. Cells exposed to \( F(ab')_2\mu\text{-Ab} \) were cultured alone or with MDA-p45\(^+\) cells as indicated. After 24 hours in culture, the cells were stained with PI and analyzed for apoptosis by flow cytometry.

Fig 6. Exposure to CD40L prevents apoptosis of BRG-M cells. BRG-M cells were cultured with MDA-p45\(^+\) cells or \( F(ab')_2\mu\text{-Ab} \) in the presence or absence of CD40L (supernatant of CD40L-CD8\text-a hybridoma, at the final dilution of 1:20). The cells were harvested after 24 hours, stained with PI, and analyzed for apoptosis by flow cytometry.
the recruitment of BL cells into the breast, a feature that characterized the clinical course of the patient.

Although the pathogenetic relevance of stimulation by exogenous or self-antigens in lymphoproliferative disorders was postulated more than 30 years ago on theoretical grounds, only recently has it received experimental support. Patients have been described in whom the malignant lymphoid cells produced antibodies or autoantibodies of defined antigenic specificity. Some of these cases were AIDS patients who developed BL or other lymphoproliferative disorders. Moreover, in a number of patients, indirect evidence of antigenic stimulation was provided by the finding that these malignant cells had features characteristic of memory cells. Based on these findings, there has been increasing recognition that stimulation by exogenous or self-antigens may act as a promoting event in the expansion of malignant clones. Moreover, observations both in humans and in experimental animal models have emphasized the role of T cells in supporting the clonal expansion of malignant B cells.

Our observations on patient BRG suggest a complex role for the stimulation of malignant cells by antigen. Although specific antigen may prolong cell survival or induce cell proliferation in other lymphoproliferative disorders, the BL studied here (and perhaps in other similar cases), the specific interaction between surface IgM and p45 caused cell apoptosis, as supported by several lines of evidence. Thus, upon coculture with MDA p45+ cells, but not with MDA p45− cells or other cells not expressing p45, BRG-M cells underwent apoptosis. This apoptosis could be blocked by a monovalent F(ab′)-ap-Ab. Moreover, BL cells other than p45- cells or other cells not expressing p45, BRG-M cells that, like most BL cells, do not express Apo1−/CD95 surface structures (data not shown), thus ruling out that apoptosis could be induced through a non-BCR mediated signal such as the Apo1-receptor pathway.

The outcome of the process initiated in vitro by the encounter between p45 and its specific receptor on the BRG-M cell surface (ie, cell death or cell rescue) appeared to be dictated by the availability of CD40L. Because CD40L is expressed on the surface of activated T cells as well as of other cells such as basophils or mast cells, the collected evidence suggests that such CD40L-expressing cells may have been involved in the pathogenesis of BRG lymphoma in vivo. Unfortunately, the paucity of circulating T cells and the rapidly deteriorating clinical conditions of the patient prevented studies on the T-cell response to p45 peptides or to other antigens from breast cells. However, the aforementioned observations of accumulation of somatic mutations in the VH gene segments encoding the antibody CDR and the isotype switch from IgM to IgA provide an indirect support for T-cell participation in the maturation of these cells and possibly in lymphomagenesis. In this regard, it is of note that IgA1-producing clones of BRG-P cell line maintained their specificity for p45 and were induced into apoptosis upon coculture with p45 expressing cells (data not shown). Thus, the process of intraclonal maturation detected in the lymphoma cells did not significantly alter their antigenic specificity, despite the apparent disadvantage imposed by the expression of a receptor capable of delivering apoptotic signals. These disadvantages were possibly counterbalanced by the advantages provided by CD40L. These advantages may have also prevented any additional form of receptor editing by the lymphoma cells.

Analyses of the c-myc rearrangement showed that the breakpoint on chromosome 8 involved the c-myc gene rather than flanking sequences, whereas the breakpoint on chromosome 14 involved the JH locus. This rearrangement likely resulted from recombinase errors before membrane Ig receptor expression. Thus, this rearrangement was an early event in the process of transformation, a finding that is also consistent with the observation that the same c-myc translocation characterized both the IgM- and IgA1-producing clones. Therefore, it appears that antigenic stimulation occurred after the major cytogenetic changes characteristic of all BL. Whether and how the presence of the rearranged c-myc facilitated the onset of an autoimmune reaction directed to p45 remains to be seen.

The present findings offer a number of clues to explain some of the phenomena occurring in AIDS patients with BL. For example, these observations support the notion that antigenic stimulation is a relevant predisposing factor for BL pathogenesis. Moreover, the requirement for the help of CD40L-expressing cells is consistent with the observation that BL may arise in HIV-seropositive patients before impairment of the immune system becomes obvious. Moreover, because CD40L-expressing cells other than T cells presumably are not deleted by HIV, there is a large window of rescue. The antigenic stimulation supporting the pathogenesis of lymphomas may involve HIV-related antigens or antigens of opportunistic microorganisms, or even self-antigens, thus reinforcing the concept that immune dysregulation caused by HIV may concomitantly induce autoimmunity and neoplasia.

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Apoptosis of Burkitt's lymphoma cells induced by specific interaction of surface IgM with a self-antigen: implications for lymphomagenesis in acquired immunodeficiency syndrome

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