Regression of Experimental Burkitt's Lymphoma Induced by Epstein-Barr Virus–Immortalized Human B Cells

By Giovanna Tosato, Cecilia Sgadari, Kazuyuki Tago, Karen D. Jones, Sandra E. Pike, Amy Rosenberg, Joan M.G. Sechler, Ian T. Magrath, Lori A. Love, and Kishur Bhatia

Epstein-Barr virus (EBV)-immortalized human B cells survive only transiently when injected subcutaneously into athymic mice, whereas Burkitt's lymphoma cells give rise to progressively growing subcutaneous tumors. In this study, we tested whether these Burkitt's tumors could be induced to regress via a bystander effect induced by EBV-immortalized B cells. Simultaneous inoculation of EBV-immortalized B cells and Burkitt's lymphoma cells in the same subcutaneous site resulted in tumors that regressed with necrosis and scarring. Similarly, simultaneous inoculation of EBV-immortalized B cells and Burkitt's lymphoma cells in separate subcutaneous sites resulted in regression of a proportion of the Burkitt's tumors. Furthermore, most of the established human Burkitt's tumors regressed with necrosis and scarring after intratumor inoculations with EBV-immortalized B cells. The EBV-immortalized B cells continued to exert this antitumor effect even when killed with irradiation. The experimental approach to Burkitt's lymphoma treatment described here exploits the ability of athymic mice to reject EBV-immortalized B cells to target an effective antitumor response to malignant cells normally incapable of eliciting it.

This is a US government work. There are no restrictions on its use.

MATERIALS AND METHODS

Cells and cell lines. EBV-immortalized cell lines were obtained either by exposure of normal peripheral blood B cells to EBV (B95-8 strain) or by spontaneous outgrowth in vitro of peripheral blood B cells from normal EBV-seropositive individuals. The Burkitt's lymphoma cell lines CA46 and JD38 were derived by spontaneous in vitro outgrowth of single cell suspended Burkitt's lymphoma tissues. All cell lines were maintained in RPMI 1640 medium (Biofluids, Rockville, MD) supplemented with 5% heat-inactivated fetal calf serum (FCS; Reheis, Armour Pharmaceuticals, Kankakee, IL), 2 mmol/L L-glutamine (GIBCO, Grand Island, NY), and 5 μg/mL gentamicin (Sigma Chemical Co, St Louis, MO). All cell lines were mycoplasma-free. Peripheral blood T cells were obtained from normal mononuclear cells by rosetting with 2-aminoethylisothiouroniumbromide–treated sheep red blood cells.

Animal studies. Six- to eight-week-old female BALB/c nu/nu mice (National Cancer Institute [NCI], Bethesda, MD) maintained in pathogen-limited conditions were used throughout. Twenty-four hours before initiation of the experiments, the mice received 400-rad total body irradiation. Exponentially growing human B cell lines with a viability greater than 95% were injected (10⁷ cells in 0.2 mL RPMI 1640 medium containing 10% FCS) subcutaneously in the right abdominal quadrant through a 25-gauge needle on plastic 1-mL syringes. In a number of experiments, two different cell lines were coinjected subcutaneously in the right abdominal quadrant (10⁷ cells from each of the cell lines in 0.1 mL RPMI 1640 medium containing 10% FCS, total injection volume 0.2 mL). In some experiments, two different cell lines were simultaneously injected in distinct subcutaneous sites, the right and the left abdominal quadrant (10⁷ cells from each cell line in 0.2 mL RPMI 1640 medium containing 10% FCS). Intratumor injections were performed by inoculation of 10⁷ cells suspended in 0.1 to 0.2 mL RPMI 1640 medium with 10% FCS into one site in the center of the tumor mass. All animals were observed twice weekly and tumor size was estimated in square millimeters as the product of two-dimensional caliper measurements (longest perpendicular length and width).

Reagents. Recombinant murine tumor necrosis factor-α (TNF-α) was a kind gift of Genentech, Inc (South San Francisco, CA); recombinant human interleukin-6 (IL-6) was a kind gift of Sandoz Pharmaceutical (Basel, Switzerland); lipopolysaccharide (LPS)
from Escherichia coli 0127:B8 was purchased from Sigma; a purified rabbit antiserum to highly purified natural murine IL-6 was a gift of Dr R. Nordan (NCI); a purified rabbit antiserum to recombinant murine TNF-α was a gift of Dr J. Weber (NCI).

Expression of surface determinants and of EBV genes. Exponentially growing human B cell lines and murine splenocytes (10⁶ cells/mL in phosphate-buffered saline [PBS] containing 5% FCS) were incubated for 30 minutes at 4°C with control reagents or monoclonal antibodies (MoAbs). These included the murine antihuman MoAbs anti-CD23 (anti-Leu 20; Becton Dickinson Immunocytometry Systems, San Jose, CA), anti-CD10 (JS; Coulter Electronics, Inc, Hialeah, FL), anti-CD18 (CLB-CD18; Janssen Biochemicals, Beerse, Belgium), anti-CD20 (anti-Leu 16; Becton Dickinson), and antitransferrin receptor (Becton Dickinson); the antismurine rat MoAbs Thy 1.2 (clone 30-H12) and CD45R (clone RA3-B2, both from Pharmingen (San Diego, CA). When appropriate, the cells were further incubated (30 minutes at 4°C) with an affinity purified, fluorescein-labeled, goat antimouse reagent (Becton Dickinson). After washing, the cells were analyzed on a FACScan (Becton Dickinson). For each determination, 10⁶ cells were examined.

The EBV-encoded nuclear proteins EBNA1 and EBNA2 were detected by Western blotting of cell lysates, using a human serum with high antibody titers to these antigens, as described.16

Cell cultures. Spleenocytes from 6- to 8-week old female BALB/c nu/nu mice were incubated for 5 days (2×10⁶ cells/mL in 24-well tissue culture plates (Limbro; Flow Laboratories, Inc, McLean, VA) in complete mouse cell culture medium consisting of 1:1 mixture of RPMI-1640 medium (GIBCO) and enriched Eagle’s medium (Biofluids, Inc, Rockville, MD) supplemented with 10% FCS (Reheis) 2 mmol/L L-glutamine (GIBCO), 10-4 mol/L 2-mercaptoethanol (GIBCO), 5 μg/mL gentamicin (Sigma), and IL-2 (500 U/mL; a gift of Cetus Corp, Emeryville, CA), as described.18 Tumors were further incubated (30 minutes at 4°C) with an antirecombinant murine TNF-α antibody (rabbit antiserum to highly purified natural murine IL-6) was a gift of Dr R. Nordan, National Institutes of Health, Bethesda, MD) in complete mouse cell culture medium.

Assays for IL-6, TNF, and interferon (IFN) bioactivities. The murine hybridoma cell line B9 (a gift of Dr R. Nordan, National Institutes of Health, Bethesda, MD) was used in a standard assay for IL-6 bioactivity.21 One unit of IL-6 bioactivity in this assay is defined as the activity inducing half-maximal lysis of B9 cells. An IL-6 concentration of 1 U/mL corresponds to approximately 20 pg of E. coli-derived human IL-6 used throughout as a laboratory standard (a gift of Sandoz Pharmaceuticals). Murine IL-6 was identified by neutralization assays with a purified rabbit antiserum to highly purified natural murine IL-6 (a gift of Dr R. Nordan, NCI). The fibroblast murine cell line L921 (a gift of Dr T. Gerrard, Center for Biologies Evaluation and Research) was used in a standard assay for TNF bioactivity.21 One unit of TNF bioactivity in this assay is defined as the activity inducing one-half maximal lysis of L921 targets. A TNF concentration of 1 U/mL corresponds to approximately 100 pg of a recombinant human TNF-α preparation (a gift of Genentech, Inc). Murine TNF-α was identified by neutralization assays with a purified rabbit antiserum to murine TNF-α (rabbit IgG antirecombinant murine TNF-α, a gift of Dr J. Weber, NCI).18 At the concentration of 20 μg/mL, this antiserum neutralized approximately 50 ng/mL recombinant murine TNF-α, as determined in the L921 bioassay.

Murine and human interferon (IFN)-induced antiviral activity were quantified by cytopathic effect neutralization assays, as previously described.22 The murine fibroblast L929 cell line was used for measuring murine IFN activity, and the human epithelial amnion cell line WISH (American Type Culture Collection [ATCC], Rockville, MD) was used for human IFN testing. One unit of IFN is defined as the reciprocal of the dilution that inhibits viral replication by 50%. Murine and human IFN reference standards were obtained from the NIH.

Cell proliferation and cytotoxicity assays. Cell proliferation was measured essentially as described.29 The Burkitt’s lymphoma cell lines JD38 and CA46 in exponential growth phase were washed and cultured in triplicate at varying cell densities (250 to 2,000 cells/well, in 96-well flat bottom plates; Costar), with or without recombinant murine TNF-α (1 to 100 μg/mL) in RPMI (1640 medium containing 2 mmol/L L-glutamine (GIBCO), 5 μg/mL gentamicin (Sigma), and 10% FCS (Reheis). After 3 and 7 days of incubation, the cultures were pulsed with 3H-thymidine (0.5 μCi/well; New England Nuclear, Boston, MA) during 4.5 hours. Results were expressed as mean cpm ± SD.

Cytotoxicity was measured using a 4.5-hour chromium release assay, as described.23 Briefly, murine leukemia YAC-1 cells (ATCC) as well as the Burkitt’s and EBV-immortalized B-cell lines, described above, in exponential growth phase were used as 3-color-labeled targets. Fresh or IL-2-activated murine spleenocytes were used as effectors at varying concentrations. The percent specific cytotoxicity was calculated as: 100 X ([experimental release – spontaneous release]/[total release – spontaneous release]).

Histology evaluation. Representative samples of tumor tissue were fixed in 10% neutral buffered-formalin (Media Kitchen; NIH), blocked in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin.

RESULTS

In initial studies (Table 1), irradiated athymic mice were injected subcutaneously with EBV-immortalized (VDS) cells alone (10⁶ cells in 0.2 mL medium), with Burkitt’s lymphoma (JD38 or CA46) cells alone (10⁶ cells in 0.2 mL medium), or with mixtures of equal numbers of EBV-immortalized (VDS) and Burkitt’s lymphoma (JD38 or CA46) cells (10⁶ cells each in 0.2 mL medium). In control experiments, 7 of 10 mice who received the EBV-immortalized B cells alone developed tumors reaching sizes of 0.25 to 2.0 cm³ that rapidly regressed, and 16 of 16 mice receiving Burkitt’s B cells alone developed progressively growing tumors, none of which regressed. In contrast, 30 of 40 mice injected with mixtures of EBV-immortalized and Burkitt’s cells developed tumors that reached sizes of 1.3 to 2.6 cm³ and then all regressed through necrosis and scarring. Of the 30 tumorbearing mice, 7 died of massive tumor-cell lysis and/or cachexia during tumor regression, but all others were cured (ie, the mice survived for at least 50 days without tumor recurrence). These experiments showed that EBV-immortalized cells induce an antitumor effect on Burkitt’s tumors in this model.

Using the same experimental system, we determined that injection of each of 4 additional EBV-immortalized B cell lines (two obtained by in vitro immortalization of normal B cells with EBV, and two by spontaneous immortalization of normal peripheral blood B cells) mixed with Burkitt’s cells (JD38 line) resulted in tumor regression in 100% of cases. In control experiments, no tumor regression occurred when CA46 Burkitt’s cells, normal peripheral blood T cells (10⁶ cells in 0.2 mL medium), or 100 ng LPS were co-injected with JD38 Burkitt’s cells (not shown). Thus, induction of Burkitt’s tumor regression appears to be a common property of EBV-immortalized B cells.

In further experiments, we examined whether the EBV-immortalized cells and the Burkitt’s lymphoma cells had to be injected into the same subcutaneous site to induce the antitumor effect (Table 2). Subcutaneous injection of EBV-
immortalized VDS cells contralateral to the Burkitt’s lymphoma JD38 cells was associated with Burkitt’s tumor development in only 7 of 15 injected mice. Of these 7 JD38 tumors, 4 underwent regression that was complete in 3 cases. In control experiments, all 7 tumors occurring in animals injected with mixtures of VDS and JD38 cells in the same subcutaneous site resulted in tumor regression, and all 4 tumors resulting from injection of JD38 cells alone progressively grew. Thus, EBV-immortalized B cells can exert antitumor effect at some distance, although perhaps at reduced levels.

Subsequently, we tested whether direct in situ injection of a growing Burkitt’s lymphoma with EBV-immortalized B cells might be able to cause regression of an already established tumor (Table 3). A single injection of 10⁷ EBV-immortalized B cells (VDS or TB lines), but not of medium control, into established JD38 Burkitt’s tumors (measuring at least 0.25 cm² in size) resulted in tumor regression in 15 of 17 cases. Of the animals with extensive necrotic tumors, 2 died, 5 were cured (ie, the mice survived for at least 50 days without tumor recurrence), and the remaining mice were killed during regression for in vitro experiments. In control experiments, all mice injected with the Burkitt’s JD38 cells alone developed progressive tumors, and all mice injected simultaneously with mixtures of JD38 and VDS cells experienced regression of all tumors. In contrast to the results with JD38 tumors, a single injection of VDS cells into established Burkitt’s CA46 tumors never induced tumor regression. However, weekly injections of VDS cells, but not of medium alone, into established CA46 tumors caused tumor regression in all 11 tumors. Of the mice with extensive necrotic tumors, 3 died, 4 were cured, and the remaining mice were killed for in vitro experiments.

In additional experiments (Table 4), we found that biweekly injections of established JD38 or CA46 Burkitt’s tumors with irradiated (15,000 rad, a dose resulting in 100% cell death after 1 week in culture) EBV-immortalized B cells (VDS, AVM, and FR lines) caused 13 of 17 tumors to regress. These experiments show that progressively growing Burkitt’s tumors can be induced to regress by intratumor injection of EBV-immortalized B cells, even if the EBV-infected B cells are growth-inhibited and expected to die because of irradiation.

Microscopic examination of regressing tumors (10 examined) showed massive central necrosis extending to the epidermis, surrounded by viable lymphoid cells of medium and large size with prominent nucleoli. Infiltration with some histiocytes was seen within and at the periphery of the tumor (Fig 1).

To elucidate the mechanism that mediates Burkitt’s lymphoma regression in this experimental system, we first examined whether the EBV-immortalized B cells cause the Burkitt’s cells to convert in vivo to a lymphoblastoid, nontumorigenic, phenotype. To this end, we compared cells from 2 JD38 and 2 CA46 regressing tumors with cells from JD38 and CA46 cell lines propagated in vitro (data not shown). Cell morphology and expression of the surface molecules, CD18, CD23, and CD10 (surface markers known to be differentially expressed on lymphoblastoid and Burkitt’s cells), did not change in Burkitt’s cells rescued from regressing tumors. Also unchanged was their tumorigenicity when injected subcutaneously into 8-week-old, BALB/c nu/nu mice (NCl) maintained in pathogen-limited conditions were total body irradiated with 400 rad and, 24 hours later, were injected subcutaneously with 10⁷ cells (in 0.2 mL RPMI 1640 containing 10% FCS) from one of three B-cell lines or with mixtures of 10⁷ cells from each of two cell lines. B-cell lines used were VDS, obtained by EBV (895-8 strain) immortalization of normal B cells, as well as JD38 and CA46, EBV-negative Burkitt’s lymphoma lines. The animals were observed twice weekly and tumor size was estimated in square centimeters as the product of two-dimensional caliper measurements (longest perpendicular length and width). Tumors were considered in regression either when two consecutive tumor measurements separated by 1 week showed a size reduction of at least 0.04 cm² or when tumors developed superficial ulcers and scarring (at least 0.25 mm² in size), indicative of tumor necrosis, without size increase.

Abbreviation: LCL, lymphoblastoid cell lines (EBV-immortalized B-cell lines).

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### Table 1. Incidence of Tumor Growth and Tumor Regression in Athymic Mice Simultaneously Injected With Burkitt’s Lymphoma Cells and B Cells Immortalized With EBV

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Lines Injected</th>
<th>Mice With Tumor</th>
<th>Days to Tumor Appearance</th>
<th>Mean Maximum Size (cm²)</th>
<th>Tumors Regressed/Total</th>
<th>Days to Regression From Day of Appearance*</th>
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<tbody>
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<td>Burkitt</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>VDS</td>
<td>JD38</td>
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<td>14.0</td>
<td>2.0</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>CA46</td>
<td>5/5</td>
<td>14.2</td>
<td>7.9</td>
<td>0/5</td>
</tr>
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<td></td>
<td>VDS + JD38</td>
<td>4/5</td>
<td>20.2</td>
<td>1.8</td>
<td>4/4</td>
<td>1/4</td>
</tr>
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<td>VDS + CA46</td>
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<td>27.2</td>
<td>2.6</td>
<td>4/4</td>
<td>2/4</td>
</tr>
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<td>JD38</td>
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<td>2/2</td>
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<td></td>
<td></td>
<td>JD38</td>
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<td>5.1</td>
<td>0/2</td>
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<td>VDS + JD38</td>
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<td>18.9</td>
<td>1.7</td>
<td>15/15</td>
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<td>VDS</td>
<td>JD38</td>
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<td>16.2</td>
<td>1.8</td>
<td>2/2</td>
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<tr>
<td></td>
<td></td>
<td>CA46</td>
<td>2/2</td>
<td>22.0</td>
<td>3.3</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>VDS + JD38</td>
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<td>35.0</td>
<td>1.3</td>
<td>3/3</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>VDS + CA46</td>
<td>4/4</td>
<td>24.0</td>
<td>2.3</td>
<td>4/4</td>
<td>1/4</td>
</tr>
</tbody>
</table>

Six 8-week-old female BALB/c nu/nu mice (NCl) maintained in pathogen-limited conditions were total body irradiated with 400 rad and, 24 hours later, were injected subcutaneously with 10⁷ cells (in 0.2 mL RPMI 1640 containing 10% FCS) from one of three B-cell lines or with mixtures of 10⁷ cells from each of two cell lines. B-cell lines used were VDS, obtained by EBV (895-8 strain) immortalization of normal B cells, as well as JD38 and CA46, EBV-negative Burkitt’s lymphoma lines. The animals were observed twice weekly and tumor size was estimated in square centimeters as the product of two-dimensional caliper measurements (longest perpendicular length and width). Tumors were considered in regression either when two consecutive tumor measurements separated by 1 week showed a size reduction of at least 0.04 cm² or when tumors developed superficial ulcers and scarring (at least 0.25 mm² in size), indicative of tumor necrosis, without size increase.

* Five of these mice were killed during regression for in vitro studies.
played effective killing of YAC cells that are exquisitely sen-
tor ratio of 100:1) by murine splenocytes from
3 separate nude mice that were either fresh or activated in
inter alia, the IL-2-activated nude murine splenocytes dis-
In additional experiments, we addressed the possibility
that the EBV-infected cells are more potent inducers of these
cells from the JD38 cell line. When a
NK functions (46% to 97% killing at an effector-to-target ratio of 100:1). Furthermore, splenocytes from 3
murine IL-6 and TNF than did progressive Burkitt's tumors. None secreted detectable levels of
survival.47 In contrast to splenocytes of a normal adult
The Burkitt's cells were mixed with the EBV-immortalized cells and then injected in one subcutaneous site (same site), or the Burkitt's cells and
The nonregressing tumor stabilized at 1.6 to 1.8 cm² over 4 weeks of observation.
* Of the four animals, two were killed when cachectic.
*** The mean maximum tumor size (cm²) at presentation, the number of tumors regressed/total, and the average number of days to regression from the day of appearance.
gested that murine TNF-α can induce Burkitt's tumors to ally grew progressively. Recombinant human IL-6 inoculation was repeated after 5 days. Five of six established Burkitt's tumors underwent macroscopic hemorrhagic necrosis 24 to 48 hours after each inoculation with TNF-α. However, no tumor regression was complete, and all tumors eventually grew progressively. Recombinant human IL-6 inoculated alone intratumorally into 4 Burkitt's tumor-bearing athymic mice (15 μg in 100 μL PBS repeated after 5 days) had no effect on tumor growth; IL-6 also had no additive or synergistic effect when administered at this dose together with TNF (5 μg repeated after 5 days). These results suggested that murine TNF-α can induce Burkitt's tumors to regress in the present model system, even though the tumoricidal effect was transient under the conditions that we used.

To further test for the possibility that TNF produced endogenously at the tumor site might be responsible for tumor regression in the present experimental system, we examined whether a neutralizing antibody to murine TNF-α might prevent or delay tumor regression. Thus, 5 8-week-old female BALB/c nu/nu mice were irradiated with 400 rad and, 24 hours later, were injected subcutaneously with 10⁷ EBV-immortalized VDS or TB cells (0.2 mL medium). The mice were injected once in experiments 1 through 3. In experiments 4 and 5, the mice were injected weekly until there was evidence of tumor regression or uncontrolled tumor progression. Animal and tumor evaluation was performed as described in the legend to Table 1.

* Days to regression were calculated from the day of inoculation of the EBV-immortalized B cells.

### Table 3. Regression of Established Burkitt's Tumors Induced by Intratumor Injections of EBV-Immortalized B Cells

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Burkitt Lines First Injected</th>
<th>Mice With Tumors/ Total Injected</th>
<th>Intratumor Injections (times reinnjected)</th>
<th>Tumor Size at First Intratumor Injection (cm²)</th>
<th>Mean Maximum Tumor Size</th>
<th>Tumors Regressed/ Total</th>
<th>Deaths During Regression</th>
<th>Days to Regression From Day of Appearance</th>
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<tbody>
<tr>
<td>1</td>
<td>JD38</td>
<td>4/5</td>
<td>Medium (1)</td>
<td>0.5</td>
<td>5.5</td>
<td>0/4</td>
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<td>—</td>
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<td></td>
<td>JD38 + VDS</td>
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<td>VDS (1)</td>
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<td>0/6</td>
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<td>CA46</td>
<td>4/4</td>
<td>Medium (1)</td>
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<td>5.9</td>
<td>0/4</td>
<td>—</td>
<td>—</td>
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<tr>
<td></td>
<td>CA46 + VDS</td>
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<td>—</td>
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<tr>
<td>3</td>
<td>JD38</td>
<td>4/5</td>
<td>Medium (1)</td>
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<tr>
<td>4</td>
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<td>Medium (3)</td>
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Six 8-week-old female BALB/c nu/nu mice were irradiated with 400 rad and, 24 hours later, were injected with one of two Burkitt's lymphoma lines, JD38 and CA46 (10⁷ cells in 0.2 mL culture medium). Either alone or mixed with 10⁷ EBV-immortalized cells. After developing a Burkitt's tumor of at least 0.25 cm², the mice were injected intratumorally with either medium alone (0.2 mL RPMI 1640 with 10% FCS) or with 10⁷ EBV-immortalized VDS or TB cells (0.2 mL medium). The mice were injected once in experiments 1 through 3. In experiments 4 and 5, the mice were injected weekly until there was evidence of tumor regression or uncontrolled tumor progression. Animal and tumor evaluation was performed as described in the legend to Table 1.

* Days to regression were calculated from the day of inoculation of the EBV-immortalized B cells.

### Table 4. Incidence of Regression of Established Burkitt's Tumors Induced by Intratumor Injections of Irradiated EBV-Immortalized B Cells

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Burkitt Lines First Injected</th>
<th>Mice With Tumors/ Total Injected</th>
<th>Intratumor Injections (times reinnjected)</th>
<th>Tumor Size at First Intratumor Injection (cm²)</th>
<th>Mean Maximum Tumor Size</th>
<th>Tumors Regressed/ Total</th>
<th>Deaths During Regression</th>
<th>Days to Regression From Day of Appearance</th>
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<td>Medium (6)</td>
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<td>VDS (7)</td>
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<td>49</td>
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<td>Irradiated VDS (5)</td>
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<td>VDS (9)</td>
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<td>4/5</td>
<td>Irradiated AVM (8)</td>
<td>0.7</td>
<td>7.9</td>
<td>4/5</td>
<td>4/4</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>CA46</td>
<td>4/5</td>
<td>Irradiated FR (10)</td>
<td>0.8</td>
<td>7.1</td>
<td>2/4</td>
<td>1/2</td>
<td>43</td>
</tr>
</tbody>
</table>

Six 8-week-old female nu/nu BALB/c mice that developed a subcutaneous Burkitt's lymphoma, induced as described previously, were injected intratumorally twice weekly with either culture medium (0.2 mL) or with 10⁷ EBV-immortalized cells from one of three B-cell lines (VDS, AVM, and FR) that were either untreated or irradiated with 15,000 rad before inoculation (injection volume, 0.2 mL). Animals and tumors were evaluated as described above.

* Days to regression were evaluated from the day of inoculation of the EBV-immortalized B cells.
Fig 1. Gross and microscopic morphology of tumors from nude mice showing representative Burkitt's lymphoma tumors in regression are shown. Female BALB/c nu/nu 8-week-old mice were injected subcutaneously with the Burkitt's cell line JD38 \(10^7\) cells. After development of subcutaneous tumors (at least 0.25 cm\(^3\)), the mice were injected intratumorally with EBV-immortalized VDS cells \(10^7\) cells. (A) Gross morphology of a regressing Burkitt's tumor showing central ulceration and scarring of the skin overlying the tumor. (B), (C), and (D) show the histopathology of a regressing Burkitt's tumor: (B) necrotic tissue on the right extending to the epidermis and adjacent viable tumor (original magnification \(\times\) 50); (C) higher power view of the tumor-necrotic tissue interface (original magnification \(\times\) 200); and (D) the tumor consisted of polymorphic populations of large immunoblastic and plasmagymphocytic cells with prominent nucleoli and occasional mitosis (original magnification \(\times\) 400). (B), (C), and (D), hematoxylin and eosin stain.

16 to 26 days after inoculation, all 5 mice were injected intratumorally with EBV-immortalized B cells (VDS cell line, \(10^7\) cells in 0.2 mL culture medium). Twenty-four hours later, 2 animals began intraperitoneal treatment with 5 mg of a neutralizing rabbit antiserum to murine TNF-\(\alpha\) (rabbit IgG antirecombinant murine TNF-\(\alpha\); 20 \(\mu\)g/mL of the antiserum neutralized 50 ng/mL recombinant murine TNF-\(\alpha\); the other 3 mice received control PBS intraperitoneal inoculations. These treatments were repeated every 2 to 3 days for 15 total inoculations. Mice that received anti-TNF-\(\alpha\) antibody experienced tumor regression 38 and 46 days after intratumor injection of VDS cells. Control mice that received PBS only experienced tumor regression 37, 46, and 52 days after intratumor injection of VDS cells. Thus, treatment with antimurine TNF-\(\alpha\) antibody did not prevent or delay Burkitt's tumor rejection induced by EBV-immortalized B cells.

DISCUSSION

In the present study, we have explored a potentially novel therapeutic approach to the treatment of experimental Burkitt's lymphoma. When injected subcutaneously into young irradiated athymic mice, most Burkitt's lymphoma cell lines give rise to progressively growing subcutaneous tumors. In the same experimental system, EBV-immortalized cell lines survive only transiently, presumably because of host immu-
Cytokine production in vitro by tumor-derived cells is shown. After removal from the animals, tumors were fractionated into single-cell suspensions and incubated for 24 hours in 6-well plates at 1.6 to 2.0 × 10⁶ cells/mL in RPMI 1640 medium supplemented with 10% FCS, 2 mmol/L L-glutamine, and 5 µg/mL gentamicin. Cell-free supernatants were tested for IL-6, TNF, and IFN bioactivity in standard bioassays. Results of IFN bioactivity reflect testing on L929 cells. Cytokine bioactivity is expressed as units per milliliter of culture supernatant conditioned by tumor-derived cells for 24 hours. Among regressing Burkitt’s tumors, the triangles identify tumors derived from simultaneous inoculations of EBV-immortalized B cells and Burkitt’s cells; the circles identify tumors in which EBV-immortalized cells were injected into established Burkitt’s tumors.

Burkitt’s tumor regression in this model system followed characteristic patterns. The tumors first developed a central scar that progressively enlarged to cover the entire tumor mass. Subsequently, the scar fell off, and new skin formed. Occasionally, small tumors regressed by progressive size reduction, without superficial scarring. Histologically, tumors with macroscopic evidence of regression showed central necrosis extending to the epidermis and variable numbers of surrounding viable-looking cells of medium to large size with prominent nucleoli. Tumor infiltration with inflammatory cells was not prominent and consisted of histiocytes both within and at the periphery of the regressing tumors.

The mechanism that mediates Burkitt’s lymphoma regression in this system has not been fully elucidated. However, several mechanisms were examined in detail. Initially, we considered the possibility that the EBV-immortalized cells induce the Burkitt’s cells to switch to a less tumorigenic, lymphoblastoid phenotype. However, Burkitt’s cells rescued from regressing tumors were indistinguishable from Burkitt’s cells maintained in vitro in the expression of those surface markers that are known to distinguish a lymphoblastoid from a Burkitt’s phenotype. They also continued to be EBV-negative and to be highly tumorigenic in athymic mice. These findings suggested that tumor regression was more likely caused by stimulation of a tumoricidal response in this host.

Although severely immunocompromised, nude mice display high levels of NK function and may develop activated monocytes in response to certain tumor cells. In addition, athymic mice may develop mature murine T cells after inoculation of human T cells, presumably because of extrathymic precursor T-cell maturation. We looked for the presence of mature T cells in the spleens of nude mice with regressing Burkitt’s tumors, but found them to be less than 1% of the splenocytes. T cells also appeared to be absent at the tumor site, because we were unable to grow IL-2-responsive cells from regressing tumors. Furthermore, subcutaneous inoculation of Burkitt’s lymphoma cells in 2 nude mice that had previously rejected the same Burkitt’s
tumor after intratumor injections with EBV-immortalized cells resulted in the appearance of progressively growing tumors (preliminary results not shown). This suggests that tumor regression in this system is not associated with T-cell memory.

In subsequent experiments, we explored the role of NK cells in Burkitt's tumor regression. However, we found that one of the Burkitt's cell lines used here, CA46, was highly resistant to killing from fresh nude splenocytes. This cell line was also resistant to killing by IL-2-activated nude splenocytes. In addition, mice with regressing Burkitt's tumors did not show increased splenocyte killing of NK-sensitive targets. Thus, it appeared unlikely that either T cells or NK cells were involved in tumor regression in the present system.

Cell suspensions derived from regressing Burkitt's tumors were found to secrete significantly higher levels of murine IL-6 and TNF-α compared with those from progressive tumors. This suggested that EBV-immortalized cells are more potent inducers of inflammatory cytokines than are Burkitt's cells, and prompted us to examine whether IL-6 and/or TNF-α might be involved in tumor regression. A direct effect of the murine cytokines on the human Burkitt's cells appeared unlikely because murine IL-6 does not bind to the human IL-6 receptor, and recombinant murine TNF-α neither inhibited the growth nor killed Burkitt's cells in vitro. However, TNF-α is known to cause intravascular thrombosis and, secondarily, tumor tissue ischemia. All the regressing tumors studied here had central necrosis occasionally surrounding a blood vessel, suggesting that tumor necrosis may be related to tissue ischemia. In addition, we observed that 5 of 6 established Burkitt's tumors underwent macroscopic necrosis after 1 or 2 intratumor inoculations of 5 μg recombinant murine TNF-α. In contrast, IL-6 was ineffective alone and appeared not to increase the tumorigenic effect of exogenous TNF-α. Although the effects of exogenous TNF-α injection were transient, it was possible that endogenously produced TNF at the tumor site, over a prolonged period of time, might have been responsible for Burkitt's tumor regression. However, multiple intraarterial injections of a neutralizing antiserum to murine TNF-α failed to appreciably affect tumor regression. Although TNF may be one of several factors involved, a role for TNF in Burkitt's tumor regression induced by EBV-immortalized B cells remains uncertain.

As discussed above, initial necrosis characterizes tumor regression in this system, suggesting that tissue ischemia may be central to this process. We now believe that factors regulating vascular endothelium growth and survival at the tumor site may be responsible for Burkitt's lymphoma's progressive growth and regression in this system. Our present understanding of the intricacies of vascular endothelium biology is still limited, but this is an increasingly active area of investigation. Elucidation of these processes is essential for understanding Burkitt's tumor growth and regression in this system.

With improved chemotherapeutic and adjuvant therapies, Burkitt's lymphoma is cured in 70% to 80% of all cases. However, B-cell lymphomas in acquired immunodeficiency syndrome, including Burkitt's lymphoma, have a median survival of 2.5 months despite therapy. Although further study will be necessary to clarify the mechanisms for the observed antitumor effects, these data support the potential clinical application of this approach for treatment of Burkitt's tumors not amenable to other treatments.

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