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Vesicular Stomatitis Virus: A Potential Therapeutic Virus for the Treatment of Hematologic Malignancy

BRIAN D. LICHTY,[1] DAVID F. STOJDL,[1] REBECCA A. TAYLOR,[1] LEIGH MILLER,[1] IRINA FRENKEL,[1] HAROLD ATKINS,[1] and JOHN C. BELL[1,2]

ABSTRACT

Certain strains of vesicular stomatitis virus (VSV) have been shown to be oncolytic in a wide variety of solid tumors. In the present study, we tested the leukemolytic properties of VSV using established leukemia cell lines and primary patient material. VSV efficiently killed essentially all leukemic cell lines. In contrast, however, normal clonogenic bone marrow progenitor cells and peripheral blood cells were remarkably refractory to infection by VSV. By exploiting this large difference in susceptibility to infection we successfully purged contaminating leukemic cells from cultures of peripheral blood progenitor cells (PBPC) using VSV. VSV was also able to infect and kill leukemic cells in primary samples taken from patients with multiple myeloma (MM). This study demonstrates the potential utility of VSV in the treatment, both *ex vivo* and *in vivo*, of hematologic malignancies.

OVERVIEW SUMMARY

We have previously proposed the use of vesicular stomatitis virus (VSV) as an oncolytic virus for the treatment of malignant disease. We have also recently reported low toxicity and excellent efficacy in mice with solid tumors after systemic delivery of interferon-inducing VSV mutants. In the present study we have examined the potential utility of VSV as a leukemolytic agent. VSV was able to efficiently kill 11 of 12 lines in a panel of human leukemia cell lines and 3 of 3 primary multiple myeloma patient samples. We also demonstrate the resistance of normal hematopoeitic cells to infection and killing by VSV. Also, in this study we have capitalized on this large difference in susceptibility to VSV to purge peripheral blood progenitor cell (PBPC) cultures of contaminating leukemia cells. Taken together, we feel that these strains of VSV are excellent candidates for the treatment of leukemia.

INTRODUCTION

THE INTERFERONS are secreted proteins discovered over 30 years ago on the basis of their antiviral properties (reviewed in Pestka *et al*., 1987). Interferon (IFN) acts to inhibit cellular replication and to induce an antiviral state in cells exposed to it (reviewed in Stark *et al*., 1998). Because of its cytostatic properties it was hoped that IFN would be a valuable cancer therapeutic. Starting in 1970 a number of clinical trials were held to determine the utility of IFN therapy in malignancy. The results of most of these trials were disappointing and today only a handful of cancers are treated with IFN. These include chronic myelogenous leukemia, cutaneous T-cell lymphoma, hairy cell leukemia, melanoma, and renal cell carcinoma (Grander and Einhorn, 1998). Most of the more common malignancies—breast, prostate, colon, lung, stomach, and uterus—do not respond to IFN therapy (Grander and Einhorn, 1998). Additionally, when tumors respond to IFN therapy the response is usually transient because IFN-resistant subclones eventually arise (Grander and Einhorn, 1998). Thus, it would appear that a loss of responsiveness to the cytostatic properties of IFN is a common component of tumorigenesis.

Vesicular stomatitis virus (VSV) is a negative-sense RNA virus. This virus has a broad host cell tropism *in vitro*. In nature, however, the virus most commonly infects farm animals with insect hosts acting as vectors for transmission (Dietzschold *et al*., 1996). Naturally occurring infections of humans are rare (Fields and Hawkins, 1967) and are generally benign (reviewed

[1]Ottawa Regional Cancer Centre Research Laboratories, Ottawa, Ontario, Canada K1H 1C4.
[2]Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario Canada, K1N 6N5.

in Lichty *et al*., 2004). A striking characteristic of VSV is the exquisite sensitivity of the virus to IFN. IFN induces an antiviral state in the host cell that severely inhibits the replication of VSV (Masters and Samuel, 1983). In fact, VSV is commonly used in the laboratory as a biologic assay for the presence of an IFN-induced antiviral state (Julkunen *et al*., 1982; Salonen and Salmi, 1982; Marquardt *et al*., 1992).

We have previously reported the extreme sensitivity of human malignant cells to infection and killing by VSV, even in the presence of IFN (Stojdl *et al*., 2000, 2003). We hypothesize that hematologic malignancies may be an attractive target for oncolytic virus therapy because the target cells should be easily accessed by the therapeutic virus. Additionally there have been many reports of mutations or deletions of members of the interferon regulatory factor (IRF) family of transcription factors in multiple myeloma (MM) and myeloid leukemia (Boultwood *et al*., 1993; Willman *et al*., 1993; Harada *et al*., 1994; Linge *et al*., 1995; Beretta *et al*., 1996; Iida *et al*., 1997; Kondo *et al*., 1997; Haus, 2000; Tzoanopoulos *et al*., 2002). Conversely, few hematologic malignancies are treated with IFN, outside of chronic myelogenous leukemia, but even there the response tends to be transient and IFN-resistant disease eventually arises (see above). The possibility that myeloid leukemias, in particular, have pre-existing or treatment selected, defects in IFN responsive genes led us to test the susceptibility of acute myeloid lymphoma (AML) cell lines to infection and killing by VSV. In the current study we further assessed the ability of VSV to infect and kill human leukemia cells. We report here the efficient killing of 11 of 12 leukemic cell lines tested. This is in sharp contrast to the remarkable resistance to VSV displayed by normal bone marrow progenitors and peripheral blood cells. We were able to purge peripheral blood progenitor cell (PBPC)/leukemia cell line mixtures of the leukemic cells, demonstrating that these leukemic cells were greater than 1000 times more sensitive to infection and killing by VSV than were the PBPC in the coculture. Additionally, we extend the sensitivity of leukemia cell lines to patient samples and demonstrate that samples from patients with MM are also sensitive to infection and killing by VSV. These results were obtained with IFN-inducing mutants of VSV that are severely attenuated *in vivo* but retain their ability to infect and kill malignant cells because of the defective response to IFN commonly seen in malignant cells (Stojdl *et al*., 2003; Lichty *et al*., 2004). This study demonstrates the utility of VSV in the treatment, both *ex vivo* and *in vivo*, of haematologic malignancies.

MATERIALS AND METHODS

Cells and viruses

Cell lines were grown in Iscove's modified Dulbecco's medium (IMDM; Gibco, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS) (Gibco). Growth factor-dependent lines (OCI/AML1, OCI/AML4, OCI/AML5, H191 and MO7e) were grown in IMDM supplemented with 10% FBS and 10% 5637-conditioned media. H191 is an AML cell line derived from patient material by H.A. (co-author). Viruses were grown in Vero cells grown on microcarrier beads in spinner flasks, concentrated on a sodium potassium tartrate isopycnic gradient and titered on Vero cells. The three viral strains used in this study are described elsewhere in detail (Stojdl *et al*., 2003). Briefly, these viruses consist of the heat-resistant (HR) strain of VSV and the IFN-inducing mutants AV1 and AV2. AV1 and AV2 are naturally occurring mutants displaying a small plaque phenotype on IFN responsive cells and have mutations in the matrix gene (M51R and V221F/S226R mutations in matrix for AV1 and AV2, respectively) (Francoeur *et al*., 1987).

Recombinant VSV

A recombinant VSV expressing enhanced green fluorescent protein (EGFP) was constructed by subcloning the EGFP open reading frame (ORF) from pEGFP-N1 (Clontech, Palo Alto, CA) into the cloning site introduced between the G and L genes of VSV in the genome vector pVSV-XN (Schnell *et al*., 1996). This recombinant genome was rescued using standard techniques (Schnell *et al*., 1996) to generate a replication competent, GFP-expressing VSV clone.

Patient samples

Three patients with MM were included in this study. Diagnosis was based on clinical examination, peripheral blood count, and the morphology and immunophenotype of the leukemic cells. Normal bone marrow cells were obtained from the allogeneic bone marrow transplant donors. Mononuclear cells were collected after centrifugation through a Ficoll gradient. Cryopreserved PBPC samples were obtained by peripheral vein leukophoresis from patients after cytokine therapy to mobilize stem cells. Peripheral blood cells were obtained from normal volunteers and nucleated cells were isolated after red cell lysis. Samples were obtained after informed consent of the patient/donor under a protocol approved by the Ottawa General Hospital Ethics Review Board.

Purging experiments

Purging experiments were carried out by spiking cultures of normal peripheral blood stem cells with leukemic cells that were not cytokine-dependent to a final concentration of 1% leukemic contamination. After infection with virus cells were plated at two serial dilutions per treatment. One dilution series was plated in methylcellulose containing 5% FBS while the other series was plated in Lite Methylcellulose plus Epo (Gencyte, Amherst, NY). Only leukemic progenitors were able to form colonies when plated in methylcellulose containing 5% FBS as pure PBPC samples failed to form any colonies. The methylcellulose cultures were examined after 10 days and the number of leukemic precursors (colony count in 5% serum) and the number of normal precursors (colony count in Gencyte methylcellulose minus colony count in 5% serum) was determined for each treatment.

Infections

Infections of suspension cells were carried out in small volumes of media at cell concentrations greater than 1×10^6/ml

for 45 minutes at 37°C with occasional mixing and then plated into culture dishes in the appropriate media.

Colony assays

Bone marrow and PBPC colony assays were performed by plating duplicate serial dilutions in Lite Methylcellulose plus Epo (Gencyte) in 35-mm dishes followed by incubation at 37°C for 10 to 14 days. Colonies were scored as CFU-E (colony forming units-erythroid) or CFU-GM (colony forming units-granulocyte/macrophage). Cell line colony assays were performed by plating duplicate serial dilutions in methylcellulose plus 10% FBS. For cytokine-dependent cell lines (see above) 10% 5637-conditioned media was included.

Flow cytometry

The viability of unfixed cells was assessed by staining with propidium iodide followed by flow cytometric analysis. Detection of infected cells was determined by enumeration of green fluorescing cells after infection with a GFP-expressing VSV. Peripheral blood populations were identified after staining with anti-CD3 (T cell), anti-CD19 (B cell), anti-CD14 (monocytes), and anti-CD13 (neutrophils). Plasma/myeloma cells in the samples from patients with MM were identified using anti-CD138 (all antibodies from Becton-Dickinson, Franklin Lakes, NJ).

RESULTS

VSV killing of clonogenic leukemia precursors

The leukemolytic properties of VSV were evaluated using a panel of leukemia cell lines and four distinct virus strains. Essentially, cells were infected at a multiplicity of infection (MOI) of 1.0 plaque-forming units per cell (pfu/cell) and then grown in a methylcellulose suspension to evaluate viability. The four different virus strains used were the heat-resistant or HR variant of VSV Indiana, a recombinant version of HR harboring the GFP gene and the IFN-inducing mutants AV1 and AV2 (Stojdl *et al*., 2003). In our earlier nude mouse studies, we had shown that while the HR strain of Indiana has good oncolytic properties, maximum therapeutic benefit was obtained when this virus was combined with IFN. On the other hand, the IFN-inducing mutants AV1 and AV2, effectively kill tumor cells lacking an IFN response but are highly attenuated for growth in normal tissues even in the absence of exogenously added IFN (Stojdl *et al*., 2003).

As can be seen in Table 1, most of the 12 cell lines tested in this way were sensitive to VSV with a reduction of as much as 5 logs in colony forming ability compared to mock-infected cultures. VSV-mediated oncolysis extended to a wide variety of leukemia types, although cell lines of lymphoid origin were generally more resistant to the virus, especially to the IFN-inducing mutants AV1 and AV2. Both the HR strain and its derivative engineered to express GFP, were equally potent at killing leukemia cells indicating that the addition of an extra gene to the virus did not markedly compromise its oncolytic activity.

Flow cytometric analysis of infected leukemia cell lines

While the colony-forming assay is a robust means of evaluating virus-mediated cell killing, it is limited. We sought a second method to evaluate VSV infection and killing of malignant hematopoetic cells. To this end we used flow cytometry following propidium iodide (PI) staining of unfixed cells to determine viability after infection (Table 2). This method allowed us to analyze cell lines that failed to form countable colonies in methylcellulose such as NCI/H929 and OCI/AML4. As ex-

Table 1. Killing of Leukemic Cell Lines by Vesicular Stomatitis Virus as Assessed by Colony Counts

	Average colony count per 10^5/cells plated				
Cell line	*Mock*	*HR*	*AV1*	*AV2*	*GFP*
LY-8 (B-cell lymphoma)	44927 ± 11930	3079 ± 2378	5560 ± 2436	3693 ± 1101	120 ± 35
LY-18 (B-cell lymphoma)	5254 ± 1740	3 ± 3	44 ± 20	311 ± 499	1 ± 1
SR (large cell lymphoma)	18738 ± 6375	0 ± 0	1 ± 1	0 ± 0	0 ± 0
MOLT-4 (acute lymphoblastic)	56977 ± 31602	104 ± 72	513 ± 71	899 ± 608	358 ± 34
Jurkat (acute lymphoblastic)	68444 ± 10584	0 ± 0	0 ± 0	0 ± 0	0 ± 0
OCI/My10 (myeloma)	35827 ± 11724	1 ± 2	58 ± 61	10 ± 8	0 ± 0
K562 (chronic myeloid)	44913 ± 17646	0 ± 0	3 ± 3	8 ± 6	0 ± 0
MO7e (megakaryoblastic)	16131 ± 5092	8 ± 7	43 ± 6	3 ± 3	1 ± 1
OCI/AML1 (acute myeloid)	32692 ± 24561	1 ± 1	1 ± 1	4 ± 3	0 ± 0
OCI/AML3 (acute myeloid)	13600 ± 6699	0 ± 0	0 ± 0	0 ± 0	0 ± 0
OCI/AML5 (acute myeloid)	66520 ± 7429	0 ± 0	0 ± 0	0 ± 0	0 ± 0
H191 (acute myeloid)	36631 ± 12552	0 ± 0	3 ± 2	14 ± 5	0 ± 0

Leukemic cells were infected with the indicated strain (HR, heat-resistant; GFP, recombinant GFP-expressing attenuated virus [AV1] and attenuated virus [AV2]) of VSV at an MOI of 1.0. Serial dilutions (10^2, 5×10^2, and 10^3 for uninfected cultures and 10^3, 10^4, and 10^5 for infected cultures) were plated in methylcellulose at 72 hr postinfection and counted ~10 days later. Mock, HR, and AV3 counts represent four independent experiments each plated in triplicate; AV1 counts derive from three independent experiments each done in triplicate and the GFP counts are from one experiment done in duplicate.

VSV, vesicular stomatitis virus; MOI, multiplicity of infection.

Table 2. Flow Cytometric Analysis of Human Leukemia Cell Lines Infected with Vesicular Stomatitis Virus

A: Lymphoid leukemia cell lines

LY-8 (B-cell lymphoma)					
	VSV (−)	HR	AV2	GFP	GFP +
24 hr	11.8	ND	12.8	55.9	49.9
48 hr	7.1	ND	58.4	66.1	47.5
LY-18 (B-cell lymphoma)					
	VSV (−)	HR	AV2	GFP	GFP +
24 hr	6.5	ND	15.2	60.6	55.1
48 hr	3.0	ND	53.7	85.1	44.7
SR (large cell lymphoma)					
	VSV (−)	HR	AV2	GFP	GFP +
24 hr	21.3	63.5	51.7	27.3	34.0
48 hr	25.1	90.2	92.1	84.5	7.1
MOLT-4 (T-cell acute lymphoid leukemia)					
	VSV (−)	HR	AV2	GFP	GFP +
24 hr	9.4	32.2	21.0	20.8	66.4
48 hr	6.7	75.9	65.5	59.8	59.5
Jurkat (T-cell acute lymphoid leukemia)					
	VSV (−)	HR	AV2	GFP	GFP +
24 hr	6.7	26.5	53.4	38.5	90.1
48 hr	11.48	84.8	98.1	93.2	94.8
OCI/My10 (myeloma)					
	VSV (−)	HR	AV2	GFP	GFP +
24 hr	20.2	ND	77.3	88.6	10.7
48 hr	17.9	ND	87.7	89.3	3.6
NCI-H929 (myeloma)					
	VSV (−)	HR	AV2	GFP	GFP +
24 hr	5.1	ND	8.4	97.4	1.5
48 hr	5.4	ND	98.9	99.1	0.0

B: Myeloid leukemic cell lines

OCI/AML1 (acute myeloid leukemia)					
	VSV (−)	HR	AV2	GFP	GFP +
48 hr	7.6	63.6	73.8	45.8	87.6
72 hr	3.1	94.0	85.6	95.4	60.1
OCI/AML3 (acute myeloid leukemia)					
	VSV (−)	HR	AV2	GFP	GFP +
24 hr	1.8	56.4	65.7	65.2	76.6
48 hr	2.1	98.9	98.5	97.3	25.3
OCI/AML4 (acute myeloid leukemia)					
	VSV (−)	HR	AV2	GFP	GFP +
24 hr	15.0	17.3	15.6	15.4	0.7
48 hr	15.5	33.4	16.3	20.5	14.4
OCI/AML5 (acute myeloid leukemia)					
	VSV (−)	HR	AV2	GFP	GFP +
24 hr	5.1	57.1	60.6	57.4	93.6
48 hr	11.7	98.4	96.1	98.1	96.7
H191 (acute myeloid leukemia)					
	VSV (−)	HR	AV2	GFP	GFP +
24 hr	7.6	27.0	41.9	32.3	87.0
K562 (chronic myeloid leukemia)					
	VSV (−)	HR	AV2	GFP	GFP +
24 hr	13.5	22.6	57.8	21.7	66.7
48 hr	ND	60.0	78.6	51.6	78.3
MO7e (megakaryoblastic leukemia)					
	VSV (−)	HR	AV2	GFP	GFP +
24 hr	27.2	44.3	46.4	60.2	89.9
48 hr	26.0	94.1	93.1	94.1	94.1
72 hr	39.0	95.1	93.0	96.4	94.7

Cell lines were infected at an MOI of 1.0, with the indicated strain of VSV, for various periods of time. Unfixed cells were stained with propidium iodide and assessed by flow cytometry. Numbers shown are percent dead or percent GFP-positive where noted. Percentages are based on 10,000 cells counted per treatment.

MOI, multiplicity of infection; VSV, vesicular stomatitis virus; GFP, green fluorescent protein; HR, heat-resistant; AV2, attenuated virus 2.

pected, there was good agreement between methylcellulose colony assay and the flow cytometric analysis, although in general, flow cytometry tended to underestimate the killing ability of the virus. In experiments using our recombinant VSV-GFP virus, it was possible to monitor the progression of the viral infection. Shortly after infection, leukemic cells began to express GFP but as the infection progressed and cells began to die (as measured by PI uptake) GFP expression was lost from some of the cell lines. For instance, OCI/AML3 and SR were efficiently killed and had lost GFP expression by 48 hr postinfection while K562 and Mo7e were also killed efficiently but they retained GFP fluorescence. The reason for this variability is not clear but perhaps reflects differences in the death pathway for these different cell types. All three strains of the virus displayed efficient infection and killing of most cell lines although the IFN-inducing mutant AV2 displayed slower growth kinetics than the wild-type strains. Overall the combination of colony assay and flow cytometry provides convincing evidence for the killing of lymphoma, acute lymphoblastic, acute myeloid and chronic myeloid leukemia cell lines.

Normal bone marrow progenitors are refractory to VSV infection

In contrast to the exquisite sensitivity of leukemic cell lines to VSV infection, normal clonogenic bone marrow progenitors are largely unaffected by the virus. As an example, in the experiments shown in Figure 1A, bone marrow from normal donors was infected with the HR-GFP strain of Indiana virus at an MOI of 5 pfu/cell and the infection allowed to proceed

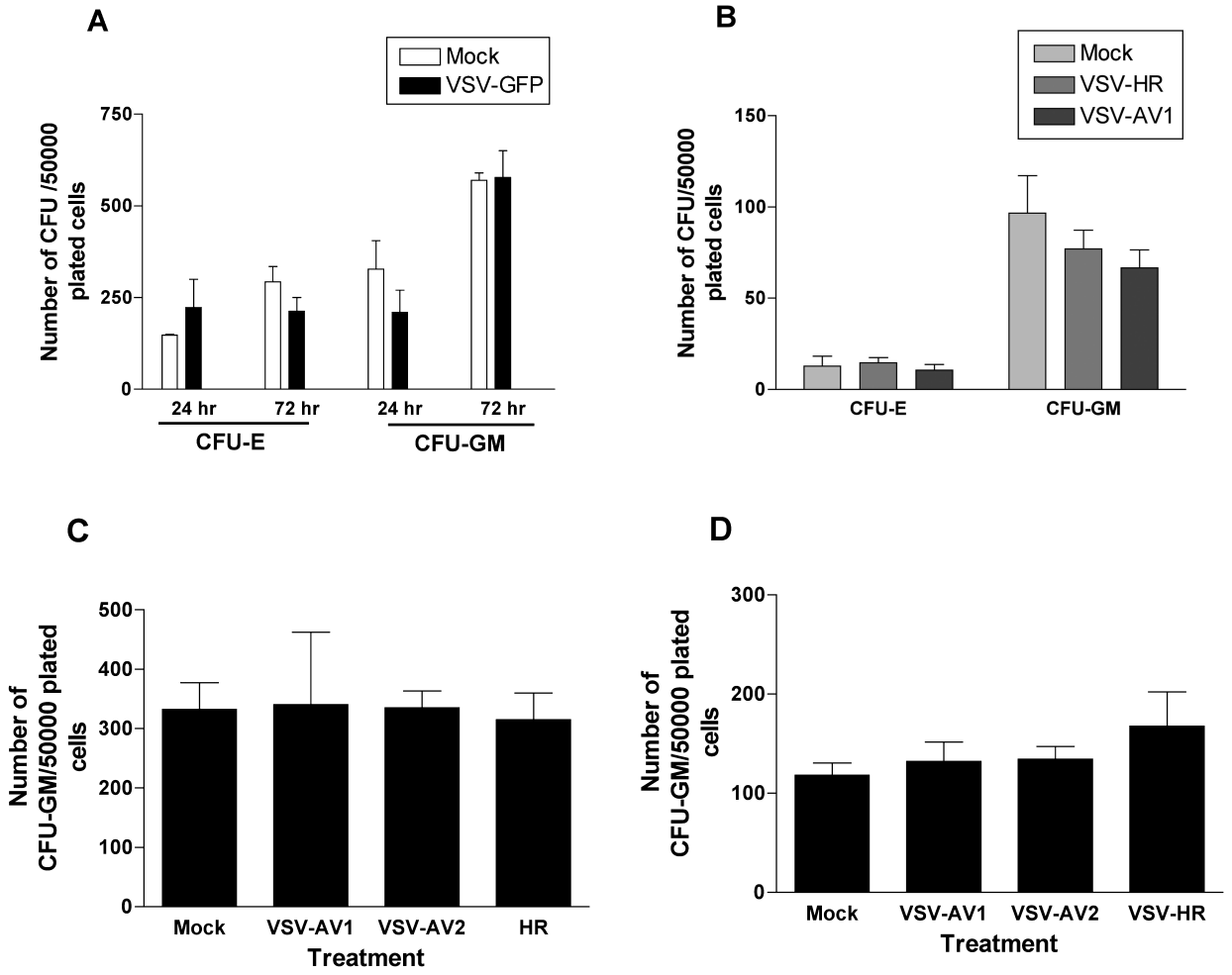

FIG. 1. Normal bone marrow progenitors are resistant to vesicular stomatitis virus (VSV). **A**: A normal bone marrow was infected at a multiplicity of infection (MOI) of 5.0 with VSV-green fluorescent protein (GFP) and at the indicated time points samples were serially diluted and plated in methylcellulose in duplicate. **B**: A second normal bone marrow sample was infected with each of the indicated viruses at an MOI of 10.0 and at 24 hr postinfection samples were serially diluted in triplicate and plated in methylcellulose. **C**: A third normal bone marrow sample was infected with each of the indicated viruses at an MOI of 1.0 and at 1 hr postinfection samples were serially diluted and plated in methylcellulose in duplicate. **D**: A fourth normal bone marrow sample was infected with each of the indicated viruses at an MOI of 1.0 and at 1 hr postinfection samples were serially diluted in triplicate and plated in methylcellulose. In each case colonies were counted after 14 days. Number of colonies shown is per 5×10^4 cells plated. CFU-E, colony forming units-erythroid; CFU-GM, colony forming units-granulocyte/macrophage.

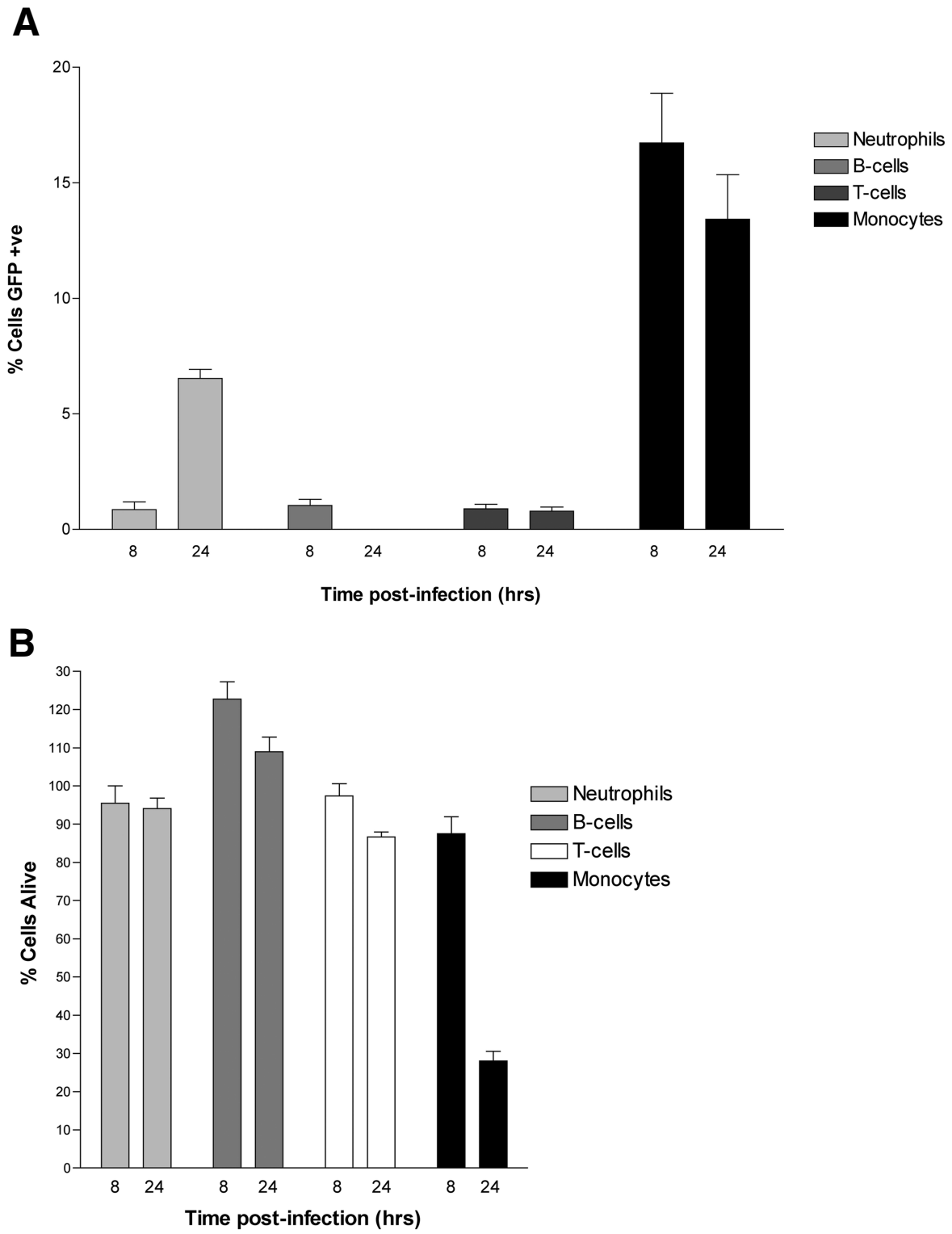

FIG. 2. Infection of normal peripheral blood cells with vesicular stomatitis virus (VSV). White blood cells were isolated from the peripheral blood of a normal volunteer and infected with VSV-green fluorescent protein (GFP) at a multiplicity of infection (MOI) of 10 plaque-forming units (PFU) per cell. **A**: Percentage of cells in each population expressing GFP at 8 and 24 hours post-infection. **B**: Viability of each subset after infection normalized to uninfected population.

for some 72 hr. At this time, the culture was plated in methylcellulose and the number of colony forming cells (CFCs) determined 14 days later. It is clear from Figure 1 that normal progenitors are unaffected by VSV infection. In a second series of experiments (Fig. 1B), using the IFN-inducing virus strains (AV1 and AV2) and the parental HR strain there was no significant difference in the plating efficiency of normal progenitors following VSV infection. Similar results were obtained for two additional bone marrow samples (Fig. 1C and 1D).

Normal lymphocytes and neutrophils are resistant to infection by VSV

To determine the degree to which normal peripheral blood cells could be infected with VSV white blood cells from five normal volunteers were infected with a recombinant VSV expressing GFP at an MOI of 10 and flow cytometry was used to determine which cell populations, if any, became infected *in vitro*. Data for a representative sample is shown in Figure 2. These analyses demonstrated that lymphocytes were very resistant to infection even at this high MOI. There was some infection of neutrophils by 24 hr (range, 4.1% to 7.9%) and monocytes were somewhat susceptible showing infection at both time points (range at 8 hr, 3.4% to 17.2% and range at 24 hr, 13.3% to 35.1%). It should be noted however that CFU-GM are resistant, therefore this susceptibility appears to be confined to mature, peripheral monocytes.

Purging of leukemia cells from mixed cultures with VSV

In the clinical setting, peripheral blood stem cells (PBSC) are used as a source of hematopoietic stem cells to perform autotransplants for patients with leukemia and contaminating leukemic cells are likely problematic (Deisseroth *et al*., 1994; Rill *et al*., 1994; Heslop *et al*., 1996). The extreme sensitivity of leukemic cells to VSV infection coupled with the remarkable resistance to the virus displayed by bone marrow progenitors led us to test the ability of the virus to purge PBPC cultures of contaminating leukemic cells. To this end, leukemia cell lines were mixed with PBPC and infected with VSV. Infected cultures were serially diluted and plated in methylcellulose containing growth factors to support normal CFCs or methylcellulose containing 5% FBS to exclusively support growth of leukemic colonies (Table 3). PBPC progenitors were also resistant to killing by the attenuated AV2 strain of VSV but were affected by infection with the HR strain. When cultures containing leukemic cells were infected with the HR and AV2 strains there was a complete ablation of leukemic cells and a

Table 3. Leukemia Cells Are More Than 1000 Times More Sensitive to Vesicular Stomatitis Virus Than Are Peripheral Progenitor Cells

	Colonies per 10^5 cells plated		
PBPC	*CFU-E*	*CFU-GM*	
Mock	282 ± 34	193 ± 48	
VSV-HR	113 ± 21	79 ± 31	
AV2	203 ± 28	165 ± 45	
PBPC plus 1% MOLT-4			
	CFU-E	*CFU-GM*	*Leukemic*
Mock	ND	ND	1140 ± 48
VSV-HR	98 ± 20	58 ± 26	0
AV2	168 ± 26	143 ± 41	0
PBPC plus 1% OCI/AML3			
	CFU-E	*CFU-GM*	*Leukemic*
Mock	ND	ND	1243 ± 50
VSV-HR	49 ± 14	40 ± 22	0
AV2	123 ± 22	112 ± 37	0

Peripheral blood from a patient treated with cytokines to mobilize stem cells was infected with VSV-HR and VSV-AV2. In addition cultures were spiked with 1% leukemia cells and infections were carried out in parallel. Cultures were infected at an MOI of 1.0 and 24 hours post-infection samples were plated in methylcellulose + 5% FBS (to enumerate leukemic colonies) or in Lite Methylcellulose plus Epo (Gencyte, Amherst, NY, to enumerate leukemic plus normal colonies, the number of normal colonies indicated represents the difference between Gencyte and 5% FBS). When mock infected the leukemia-contaminated cultures produced copious colonies in the presence of cytokines making the number of normal colonies present difficult to accurately enumerate.

VSV, vesicular stomatitis virus; HR, heat-resistant; AV2, attenuated virus 2; MOI, multiplicities of infection; FBS, fetal bovine serum.

variable but relatively small reduction in progenitor numbers. There was a reduction in PBPC progenitors of less than 50% when the cocultures were infected with AV2 but a greater than 1000-fold reduction in leukemia progenitors. The apparently greater impact of the virus on normal progenitors in the context of these mixed cultures may have been the result of replication of the virus in the leukemic cells or an indirect effect of the leukemic cells dying in these cultures. These experiments clearly demonstrate that VSV is able to specifically kill leukemic cells in mixed cultures with relatively minimal impact on normal progenitor stem cells.

VSV kills myeloma cells from primary patient samples

In order to test the ability of VSV to infect and kill primary malignant cells we obtained peripheral blood samples from three patients with MM. We chose patients with MM because we had access to several samples, we had seen good killing of myeloma cell lines, and the expression of CD138 by myeloma cells made the malignant cell population easily identified by flow cytometry. Samples from these patients were infected with wild-type or IFN-inducing mutant VSV strains and the cultures were watched for 8 days postinfection. Figure 3 demonstrates the reduction in viable $CD138^+$ cells after infection with any of these three VSV strains. While each patient had a high number of $CD138^+$ cells in their peripheral blood infection with VSV was able to completely ablate these cells in one patient sample and significantly reduce the number of $CD138^+$ cells in the other two patient samples following a single dose of VSV. Taken together these analyses indicate that VSV may be a useful therapeutic in multiple myeloma.

DISCUSSION

In earlier studies, we reported the increased sensitivity of malignant cells to infection and killing by VSV. This virus is extremely sensitive to the innate IFN mediated antiviral response, which appears to be commonly dysregulated in cancerous cells. Here, we have extended this analysis to a range of leukemic cell lines and to primary leukemia patient samples. We demonstrate that most leukemic cell lines tested are highly susceptible to infection and killing by VSV, including IFN-inducing mutants that are attenuated *in vivo* (Stojdl *et al*., 2003). In these and other experiments we have been struck by the extreme resistance of normal bone marrow progenitors to VSV infection. While in our hands, VSV is able to productively infect most cell types, it is clear that bone marrow stem cells and normal peripheral blood lymphocytes have active antiviral programs that rapidly blunt virus infections. Indeed in other studies, we have found that bone marrow stem cells resist infection by laboratory strains of reovirus, Newcastle disease virus, measles virus, and mumps virus (data not shown). The susceptibility to infection displayed by normal monocytes is not surprising as these cells have been demonstrated to be susceptible to viral infection by others (Jahn *et al*., 1999) and may be important in antigen presentation by these cells. The fact that the

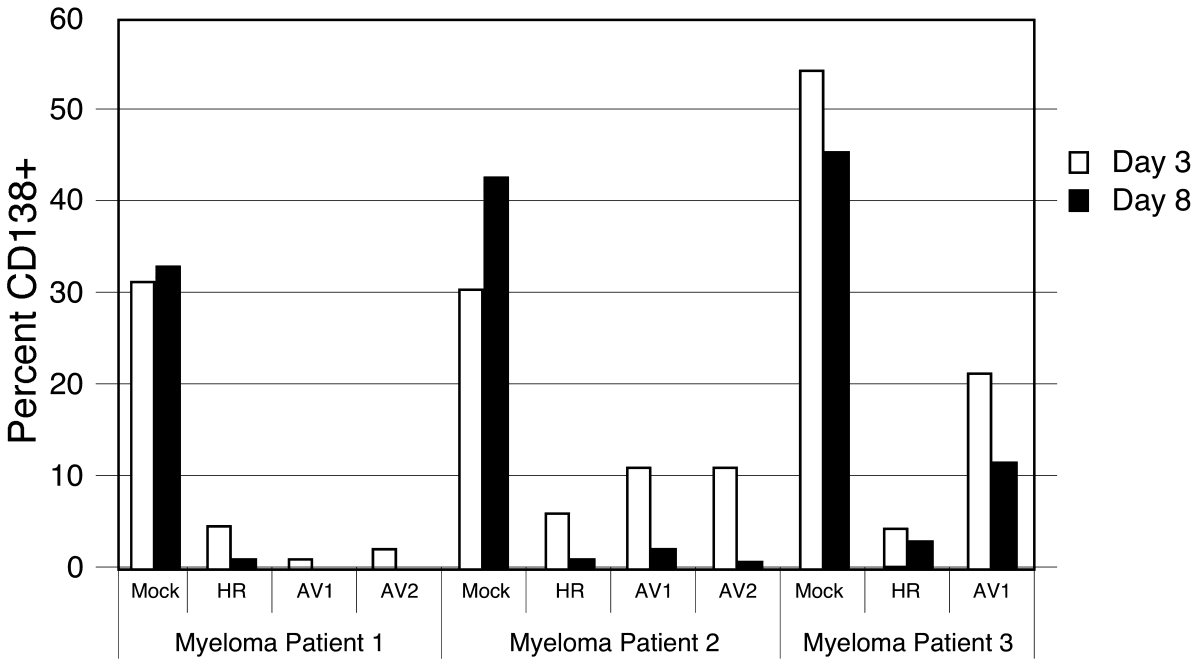

FIG. 3. *In vitro* infection and killing of samples from patients with primary multiple myeloma. Peripheral blood samples from 3 patients with multiple myeloma were infected *in vitro* with the indicated strains of vesicular stomatitis virus (VSV) at a multiplicity of infection (MOI) of 10 plaque-forming units (PFU) per cell. Samples were collected 3 and 8 days postinfection and analyzed by flow cytometry after staining for CD138. 10,000 events were recorded per sample per time point; percentages of viable cells that were CD138 positive are indicated.

progenitors for this mature cell population, namely normal CFU-GM, are resistant to VSV suggests that this cell population will be replaced by the patient's bone marrow after treatment.

The results presented here suggest that a common feature of leukemic cells appears to be the loss of at least portions of an innate antiviral response. In support of this idea, are a number of reports of defects, deletions and mutations to genes involved in the IFN response in myeloproliferative disease. For instance, IRF-1 is known to have the properties of a tumor suppressor gene in that while it plays a pivotal role in the normal IFN response to virus infection, (reviewed in Tanaka and Taniguchi, 2000) it is located at 5q31, a region frequently deleted in myeloid leukemias (Van den Berghe *et al*., 1985; Boultwood *et al*., 1993; Willman *et al*., 1993). Similarly, mutations to the PKR gene (Beretta *et al*., 1996; Abraham *et al*., 1998) have been reported in hematologic malignancies. As well, dysregulation of MUM1/IRF4 has also been reported in hematologic malignancy, particularly in MM (Iida *et al*., 1997; Yoshida *et al*., 1999; Chesi *et al*., 2000; Tsuboi *et al*., 2000; Yamada *et al*., 2001). Consistent with these observed aberrations in the IFN signaling pathway is the fact that only a handful of hematologic malignancies respond to IFN therapy (Grander and Einhorn, 1998) and that these frequently evolve to nonresponsive cancers.

The samples from patients with MM examined in this study displayed slower kinetics of killing after viral infection than the established cell lines examined although the degree of killing of primary leukemia cells is comparable to that reported for oncolytic adenovirus (Medina *et al*., 1999) and reovirus (Alain *et al*., 2002; Thirukkumaran *et al*., 2003). As well, it should be noted that the degree of killing appears to be underestimated when assayed by flow cytometry at a given time point. We are currently acquiring additional patient samples in order to determine whether MM samples are generally sensitive to VSV and to determine whether other leukemias, particularly AMLs, are also susceptible. Importantly, the enormous difference in susceptibility to VSV displayed by normal progenitors and leukemic cells suggests that the virus can be used to selectively kill leukemic cells in a mixed population. Our purging experiments support this notion. We are continuing these studies in order to determine whether there are particular classes of myeloid leukemia wherein VSV therapy might be particularly effective. We are also interested in the susceptibility of MM cells to infection and killing by VSV (and other oncolytic viruses), which is in sharp contrast to the complete resistance to infection displayed by most other lymphoid cells. Indeed this study may indicate that there is a defect in antiviral pathways in MM cells that render them particularly sensitive to infection and killing by VSV.

There have been concerns voiced by some regarding the use of replicating viral vectors in the clinical setting. We have shown that the IFN-inducing mutants used in this study are significantly attenuated *in vivo* and that they induce a cytokine cloud in the host animal effectively protecting the recipient from any wild-type revertants present in the innoculum (Stojdl *et al*., 2003). Several groups are now proposing to use VSV as a therapeutic virus and preclinical testing combined with a large body of historical data regarding natural infections of humans with this virus indicate that even the wild-type strains of this virus generally produce benign infections in naïve humans (reviewed in Lichty *et al*., 2004).

The field of oncolytic virus therapy is expanding rapidly (Bell *et al*., 2002) but in most cases these therapies are directed against solid tumors. However, efficient killing of leukemia and lymphoma cells has now been demonstrated for measles (Grote *et al*., 2001; Peng *et al*., 2001), adenovirus (Medina *et al*., 1999; Strair *et al*., 2002), reovirus (Alain *et al*., 2002; Thirukkumaran *et al*., 2003) and VSV (Stojdl *et al*., 2000; Dummer *et al*., 2001). In particular the degree of killing reported here for MM patient samples compares favourably with that seen for oncolytic measles and reovirus (Peng *et al*., 2001, 2003; Thirukkumaran *et al*., 2003). VSV is able to infect virtually all cell types but its replication is blocked in cells that have an intact innate antiviral system, especially if these cells have been exposed to IFN. Taken as a whole these observations and the results reported in this study point to the utility of VSV as a leukemolytic agent for the *in vivo* and *ex vivo* treatment of hematologic malignancy.

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Address reprint requests to:
Dr. John Bell
Ottawa Regional Cancer Centre Research Laboratories
503 Smyth Road
Ottawa, Ontario, K1H 1C4
Canada

E-mail: jbell@ohri.ca

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