

Effects of radiation and latent virus on immune responses in a space flight model

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Background: The immunosuppressive effects of space flight radiation and reactivation of latent virus infections in human beings are largely unknown.

Objective: To develop a murine model that can predict the adverse effects of space flight radiation and reactivation of latent virus infection for human beings.

Methods: In experiment I, some BALB/c mice received whole-body γ -irradiation (3 Gy) on day 0 and murine polyoma virus (PyV) on day 1. In experiment II, mice received irradiation (3 Gy) or none on days 0 and/or 49, and PyV or none on day 1: A1, 3 Gy/PyV/3 Gy; A2, 3 Gy/PyV/0 Gy; B1, 0 Gy/PyV/3 Gy; B2, 0 Gy/PyV/0 Gy; C, 3 Gy/0 Gy/PyV/0 Gy; and D, 0 Gy/0 Gy/PyV/0 Gy.

Results: In experiment I, PyV was detected by PCR more frequently in several host organs tested and for a longer period of time in irradiated than in control animals. In experiment II, PyV replication in the spleen was detected in A1>B1 mice on days 10 and 20; both groups cleared PyV by day 49. After irradiation on day 49, reactivated PyV was detected in more B1 than A1 mice. A1 mice had lower spleen weights and cell counts than other groups at all time points. From 0 to 49 days, irradiation suppressed spleen cell proliferation to concanavalin A in all irradiated groups except in B1 when the virus was cleared at day 20. PyV enhanced IFN- γ production in all groups: B1>A1>C, D (0-49 days; all differences, $P < .05$).

Conclusion: This small animal model of space flight suggests that the combined effects of radiation and virus replication will significantly affect T-lymphocyte-mediated immunity that may lead to chronic viral infection and malignancy. (*J Allergy Clin Immunol* 2005;115:1297-303.)

Key words: Space flight, radiation, polyoma virus infection, T lymphocytes, IFN- γ , virus reactivation

Long-term interplanetary space flight, such as the journey to the planet Mars envisioned by the National

Abbreviations used

HAU: Hemagglutinating unit

LSD: Least significant difference

PyV: Polyoma virus

Aeronautics and Space Administration within the next few decades, poses substantial health risks for human beings.¹⁻⁵ Many space flight conditions as tested in human and animal space travelers and in earthbound human and animal models of space flight give ample evidence of compromised immunity and the inability to resist microbial infections.⁶⁻¹⁸ Perhaps the most serious physical threat to health in space is radiation that is composed of photons (x-rays), electrons (γ -rays), protons, neutrons, and heavy metal ions.¹⁹ Investigations of mice given 3 Gy radiation (either protons or γ -rays) demonstrated by days 4 to 10 after irradiation statistically significant decreases in CD19⁺ B cells, CD3⁺ T cells, CD4⁺ T cells, and CD8⁺ T cells and IL-2 secretion by activated spleen cells.²⁰⁻²⁴ Irradiated animals showed a delayed and lowered T-cell-dependent anti-sheep red blood cell antibody response. B cells recovered by day 15 and CD3⁺ and CD4⁺ T cells by day 29, but CD8⁺ T cells remained impaired.

Reactivations of latent viruses from within the host pose a formidable threat when host immunity is compromised.^{5,25-28} Latent virus reactivation and subsequent shedding have been shown to increase in human beings in space shuttle flights (EBV; cytomegalovirus) and in the Antarctic winter-over model of space flight (polyoma virus [PyV], herpes virus)^{11-14,16} (Butel, unpublished data, 2005). These viruses are known to be associated with human and animal cancers.²⁹⁻³⁴

The hypothesis being addressed in our studies reported here is that space flight radiation will suppress the human immune system, leading to reactivation of latent viruses, increased viral infections and disease, and the development of cancers. Because we can never purposefully test this hypothesis in human beings, we use a novel murine animal model of γ -ray and PyV infection to determine harmful effects on the immune system and resultant reactivation of latent virus infection. Thus, by combining 2 space flight conditions into a quantitative animal model, we can better estimate and define the acute and long-term health risks of human astronauts.

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METHODS

Virus, animals, and infections

Murine PyV wild-type strain A2 stocks were prepared in mouse NIH3T3 cells (obtained from T. Benjamin, Harvard Medical School). Virus was titered by the hemagglutination method using guinea pig red blood cells (Colorado Serum Co, Denver, Colo).³⁵

Female BALB/c mice (6 to 8 weeks old) were purchased from Harlan Sprague Dawley (Indianapolis, Ind). Mice were inoculated intraperitoneally with 75 hemagglutinating units (HAU) of PyV in an inoculum size of 0.1 mL or an equal volume of PBS. Animals were housed in the biohazard facility at the Center for Comparative Medicine at Baylor College of Medicine, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. The studies were approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine, and animals were maintained in compliance with relevant federal regulations, guidelines, and policies.

Mice were given 3 Gy whole-body γ -irradiation by using a Gammacell Irradiator (cesium-137 source; Atomic Energy of Canada, Ltd, Ottawa, Ontario, Canada). A pilot experiment with Dr Daila Gridley at Loma Linda University (Loma Linda, Calif) revealed that proton irradiation and γ -irradiation had similar immunosuppressive effects on the murine immune response, confirming previous observations.²¹ At various times postinoculation, mice were killed and tissues were collected for analysis.

Two experiments were performed. Experiment I involved 40 animals (Table I). Experiment II involved 168 animals (Table II; Figs 1, 2).

DNA extractions and PCR analysis

Tissues were weighed and extracted for DNA. Total cellular DNA was extracted by overnight incubation of the tissue in a proteinase K solution at 55°C followed by phenol chloroform extraction and isopropanol precipitation. The DNA pellet was resuspended in Tris-EDTA buffer (pH 8.0), the DNA concentration determined spectrophotometrically, and the DNA stored at -20°C at a final concentration of 1 μ g/ μ L.

PCR primers were directed against the PyV regulatory region that gave a 442 base-pair product: JLPyVRR1-5' cacttactgtacagcttcagaagatggcgg (number 5015-5045) and JLPyVRR2-5' gaaatgagcggggaaggcgggtgg (number 159-135; nucleotide numbering according to PyV, strain A2; GenBank accession number NC-001515). Primers were obtained from Integrated DNA Technologies, Inc (Coralville, Iowa). Each PCR reaction contained 2 μ g tissue DNA (approximately 3×10^5 cell equivalents). The positive control was plasmid DNA containing a cloned PyV strain A2 genome. Negative controls for PCR assays were reactions without added DNA template. PCR amplifications were performed with a Perkin-Elmer GeneAmp PCR system 2400 thermocycler (Perkin-Elmer, Norwalk, Conn). High-stringency annealing temperatures (55°C) and 45 cycles of amplification were used. PCR amplification products were analyzed by agarose gel electrophoresis following standard protocols. The limits of detection of the PCR assay for PyV were approximately 10,000 genome copies.

Immunological studies

At various times postinoculation with PyV, mice were euthanized and body weight determined before necropsy. At necropsy, an incision of the abdominal cavity was made and the spleen surgically removed under sterile conditions. The spleen was weighed, dissected with scissors, and pressed through a sterile, stainless steel wire mesh (60 gauge, 80 mesh) with a sterile pestle. The splenic cells were suspended in 5 mL ice-cold RPMI-1640 medium, and the erythrocytes were lysed by hypotonic shock. The splenic cell suspension

was washed before viability and cell count were determined. Splenocyte suspensions were seeded in 96-well round-bottom microtiter plates at a density of 2×10^5 cells per well containing either no mitogen, LPS (16 μ g/mL), or concanavalin A (10 μ g/mL) for 72 hours. Proliferation of splenic cells was determined by the amount of [³H]-thymidine incorporated into DNA over the period of the last 16 hours of the 72-hour incubation period. The IFN- γ production by spleen cells stimulated with LPS or concanavalin A for 48 hours was measured in culture supernatants by Luminex Multiplex Bead Array assay for the detection of soluble cytokines (Luminex Corp, Austin, Tex). The assay was performed in duplicate according to the manufacturers' specifications, and a standard curve for IFN- γ was constructed by using the antibody bead kit and reference cytokine concentrations supplied by BioSource International (Camarillo, Calif). Raw data (mean fluorescent intensity) were analyzed by MasterPlex Quantitation Software (MiraiBio, Inc, Alameda, Calif) to obtain concentration values.

Statistical analysis

Significant differences in body and spleen weight, proliferation, and IFN- γ production by spleen cells activated with concanavalin A were determined among groups by using 1-way ANOVA, followed by the least significant difference (LSD) test to determine differences between 2 groups. A *P* value of <.05 was considered to be statistically significant; SPSS software version 11 (SPSS Inc, Chicago, Ill) was used in these analyses.

RESULTS

Effect of radiation on acute infection by murine PyV

In experiment I (Table I), young adult BALB/c female mice were inoculated with 75 HAU of PyV on day 1, including some animals that had been irradiated (3 Gy) 1 day earlier. Multiple tissues (kidney, liver, bone, salivary gland, lung, mammary gland, and skin) were harvested 3, 7, 12, 24, and 30 days postinoculation, were processed as described in Methods, and were assayed for viral DNA by PCR. The percentage of tissue specimens that contained detectable virus sequences was higher from irradiated mice compared with control, nonirradiated mice at each time point. Virus was cleared to undetectable levels before day 30 by control animals but not by the irradiated mice.

This experiment revealed that selected tissues appeared to support viral infection better than others. High virus tissues were kidney, liver, and bone, whereas low virus tissues were salivary gland, lung, mammary gland, and skin. The persistence of virus in irradiated mice was evident in both groups of tissues.

Spleens were harvested from these same experimental animals and were processed as described in Methods for immunological measurements. There were significant differences between the PyV-treated mice (*n* = 2) and PyV + 3 Gy-treated mice (*n* = 6) at days 3, 7, and 12. For example, at day 12, observed differences were as follows: (1) spleen weight 140 mg $\pm$ 20 mg (mean $\pm$ SEM) for PyV versus 110 mg $\pm$ 10 mg for PyV + 3 Gy (*P* = .004); (2) lymphoproliferation to concanavalin A 59,139 cpm $\pm$ 10,373 cpm for PyV versus 13,539 cpm $\pm$ 1369 cpm for PyV + 3 Gy (*P* = .001); (3) lymphoproliferation to LPS 12,470 cpm $\pm$ 1034 cpm for PyV versus 2601

TABLE I. Effect of radiation on clearance of murine PyV after acute infection of BALB/c mice

Days postinoculation*	Number (%) tissue samples PyV ⁺ †					
	Total tissues‡		High virus tissues (kidney, liver, bone)		Low virus tissues (salivary gland, lung, mammary gland, skin)	
	Not irradiated	Irradiated§	Not irradiated	Irradiated	Not irradiated	Irradiated
3	7 (50)	32 (76)	5 (83)	17 (94)	2 (25)	15 (62)
7	7 (50)	26 (62)	6 (100)	18 (100)	1 (12)	8 (33)
12	1 (7)	30 (71)	1 (17)	15 (83)	0 (0)	15 (62)
24	2 (14)	23 (55)	2 (33)	14 (78)	0 (0)	9 (38)
30	0 (0)	9 (21)	0 (0)	7 (39)	0 (0)	2 (8)

*Mice were inoculated with 75 HAU PyV intraperitoneally on day 1 of experiment.

†Detection of PyV was performed with a PCR assay (see Methods).

‡Tissues (n = 7) analyzed per mouse: kidney, liver, bone, salivary gland, lung, mammary gland, and skin. The spleen was harvested for immunological studies. Number of mice/time point: not irradiated = 2; irradiated = 6.

§Mice were irradiated with γ -rays (3 Gy) with a ¹³⁷Cs source on day 0.

||High virus tissues were defined as those for which $\geq 50\%$ of specimens from control (nonirradiated) mice collected over the period of the first 7 days postinoculation were virus-positive; low virus tissues, $< 50\%$ of specimens from controls were virus-positive.

TABLE II. Effect of γ -irradiation on replication of PyV in mouse spleen cells harvested from BALB/c mice

Day	Experimental group					
	PyV inoculated (day 1)				Controls§	
	A1	A2	B1	B2	C	D
0	Irradiated	Irradiated	Not irradiated	Not irradiated	Irradiated	Not irradiated
3	ND	ND	5/6	ND	ND	ND
10	5/6	ND	3/6	ND	0/2	0/2
20	3/6	ND	0/6	ND	0/2	ND
49	0/6	ND	0/6	ND	0/2	0/2
54	Irradiated	Not irradiated	Irradiated	Not irradiated	ND	ND
59	0/6	0/6	0/6	0/6	ND	ND
69	2/6	0/6	5/6	0/6	ND	ND
	1/6	0/6	1/6	0/6	ND	ND

Groups of mice (n = 6) were irradiated with γ -rays (3 Gy) with a ¹³⁷Cs source on days 0* and 49† and were infected with 75 HAU of PyV on day 1‡ (see key below). Detection of PyV replication and reactivation was made with a PCR assay (see Methods). The number of animals per group testing positive for viral DNA in spleen cells is indicated as a ratio for each time point.

ND, Not done.

A1, 3 Gy*/PyV‡/3 Gy†; A2, 3 Gy*/PyV‡/0 Gy; B1, 0 Gy /PyV‡/3 Gy†; B2, 0 Gy /PyV‡/0 Gy; C, 3 Gy*/PBS/0 Gy†; D, 0 Gy /PBS/0 Gy.

§In uninfected control groups C and D, 6 mice per time point were harvested for immunological studies, but only 2 animals at selected time points were tested for PyV.

cpm $\pm$ 894 cpm for PyV + 3 Gy ($P < .001$); (4) concanavalin A stimulated IFN- γ production 1891 pg/mL $\pm$ 466 pg/mL for PyV versus 789 pg/mL $\pm$ 493 pg/mL for PyV + 3 Gy ($P = .033$); and (5) LPS stimulated IFN- γ production 513 pg/mL $\pm$ 122 pg/mL for PyV versus 88.3 pg/mL $\pm$ 40.1 pg/mL for PyV + 3 Gy ($P = .001$). Some isolated spleen cells were assayed for PyV; the spleen appeared to be a high virus organ (data not shown).

Delayed clearance of primary viral infection and reactivation of latent polyoma virus infection in spleen cells after γ -radiation

In experiment II (Table II), young adult mice were inoculated with 75 HAU of PyV on day 1; some animals had been irradiated (3 Gy) 1 day earlier (groups A1, A2), whereas others had not (groups B1, B2). Control animals

that were not virus-infected were either irradiated on day 0 (group C) or not (group D). At day 49, some animals received 3 Gy radiation (groups A1, B1), whereas others were not irradiated (groups A2, B2). Animals were killed on days 3, 10, 20, 49, 54, 59, and 69. Spleens were harvested and processed for immunological assays, and a fraction of the isolated spleen cells assayed for viral DNA. Animals irradiated on day 0 (group A1) displayed elevated virus replication on days 10 and 20 compared with nonirradiated mice (group B1) on the basis of the percentage of mice with PCR-positive samples. Both irradiated and nonirradiated mice cleared the active virus infection to undetectable levels by day 49, with residual virus assumed to have established a latent state. After irradiation on day 49 (groups A1, B1), virus became detectable in the spleen by day 59, indicative of virus reactivation and replication (group B1 > group A1). In contrast, groups A2 and B2 (which were not irradiated

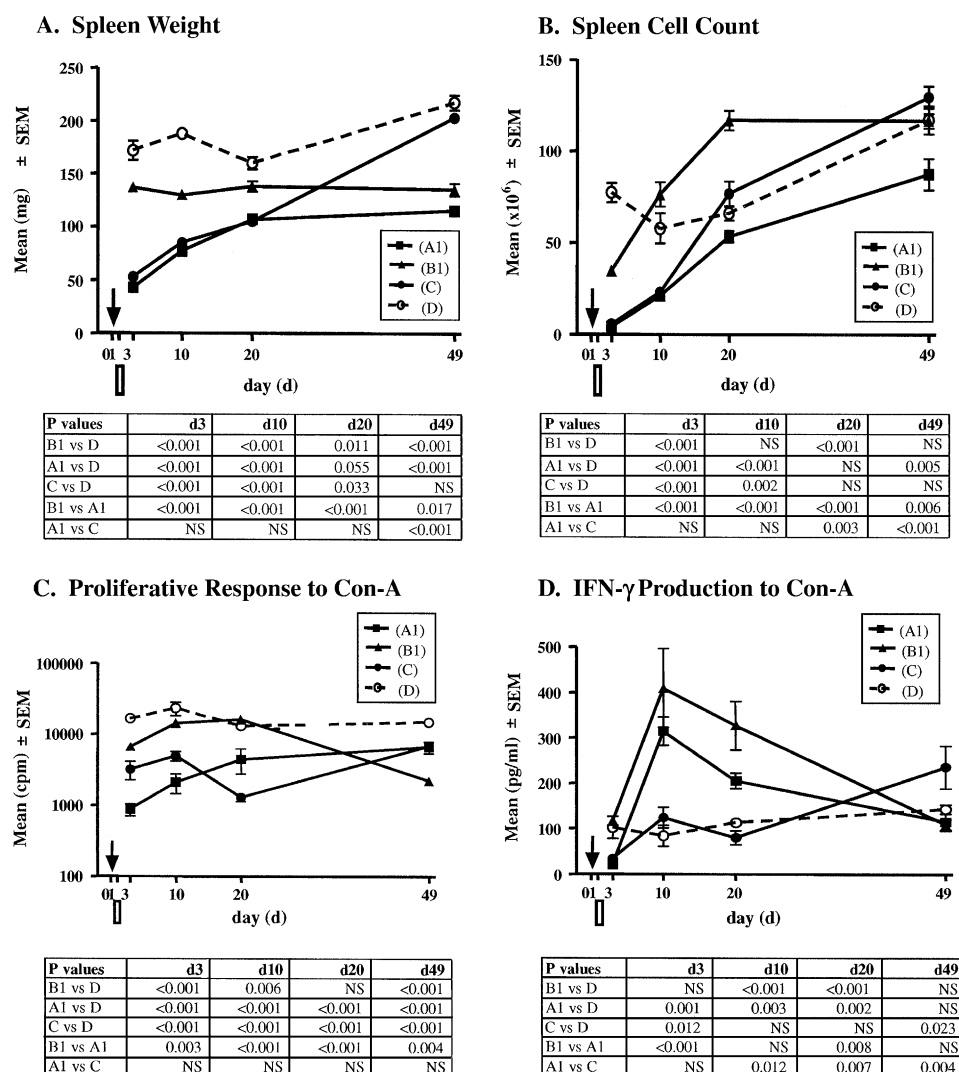


FIG 1. Effects of γ -irradiation (day 0, arrow) and primary PyV infection (75 HAU; day 1, open box) on spleen weights (A), spleen cell counts (B), proliferative responses to concanavalin A (Con A) stimulation (C), and IFN- γ production in response to Con A stimulation (D). Significance factors for differences between groups of mice (key in box in each panel) analyzed by ANOVA-LSD are given in a grid below each panel. The treatments for each group of mice (A1, B1, C, D) are described in Table II. There were 6 animals/group/time point.

on day 49) did not demonstrate virus reactivation and replication. Kidney specimens were virus-positive from animals in group A1 on days 3 (1/6) and 10 (3/6) and in group B1 on day 3 (1/6); a salivary gland sample from group A1 on day 10 (1/6) had detectable viral DNA.

Effects of γ -irradiation and primary PyV infection on splenic cells and function

Splenic weights. Radiation alone (group C) and PyV infection alone (group B1) significantly reduced spleen weights compared with control mice (group D; Fig 1, A). The combination of radiation and PyV infection (group A1) prevented recovery from the effects of radiation alone (group C) at day 49 and was more deleterious than PyV alone (group B1) at all time points.

Spleen cell counts. Radiation alone (group C), compared with the control (group D), dramatically reduced spleen cell counts by 98%, with recovery by day 20 (Fig 1, B). PyV infection alone (group B1), compared with the control (group D), significantly reduced spleen cell counts only at day 3, with recovery to normal counts at day 10 and increased spleen cell counts at day 20, with return to normal at day 49. The combination of radiation and PyV infection (group A1) significantly reduced spleen cell counts, compared with the control (group D) at days 3, 10, and 49, to radiation alone (group C) at days 20 and 49, and to PyV infection alone (group B1) at all time points.

Proliferative response to concanavalin A. Radiation alone (group C), compared with the control (group D), significantly reduced tritiated thymidine incorporation at

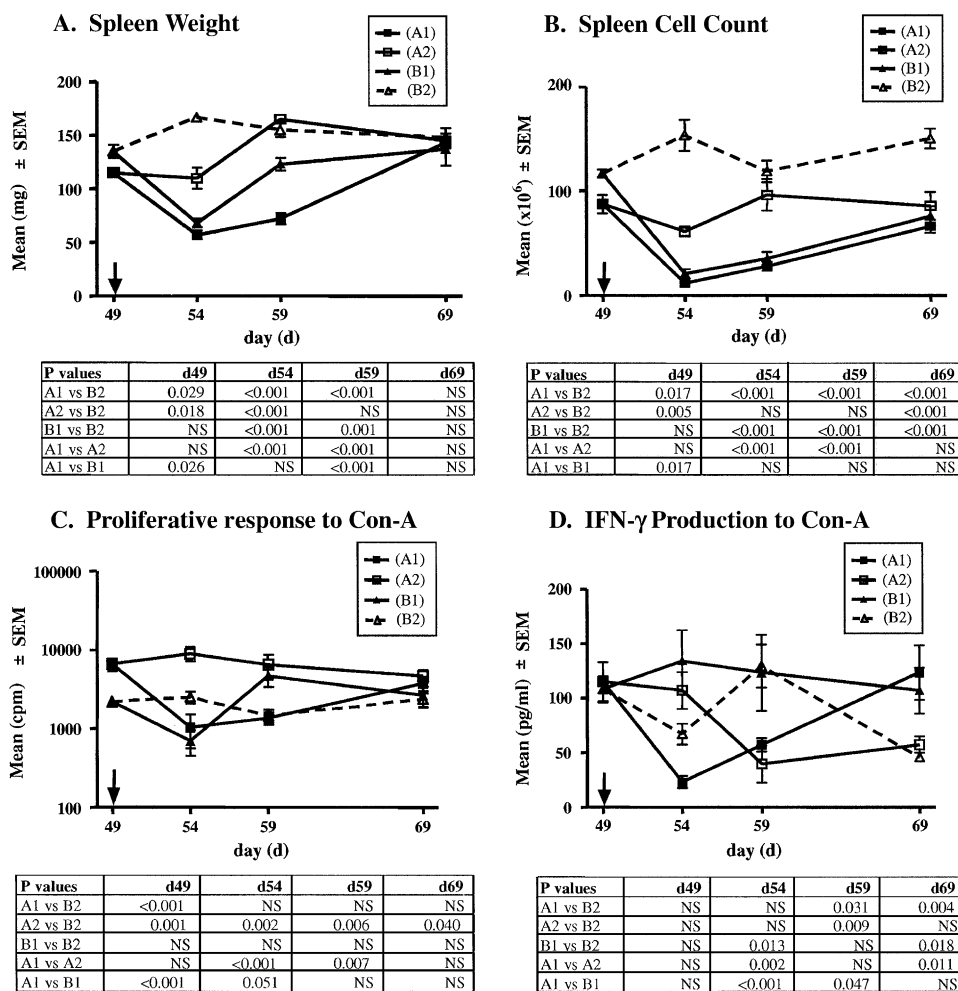


FIG 2. Effects of γ -irradiation (day 49, arrow) and reactivated PyV infection on mouse spleen weights (**A**), spleen cell counts (**B**), proliferative responses to concanavalin A (*Con A*) stimulation (**C**), and IFN- γ production in response to Con A stimulation (**D**). Significance factors for differences between groups of mice (key in box in each panel) analyzed by ANOVA-LSD are given in a grid below each panel. The treatments for each group of mice (A1, A2, B1, B2) are described in Table II. There were 6 animals/group/time point.

all time points (Fig 1, C). PyV infection alone (group B1), compared with the control (group D), significantly reduced the amount of radioactivity incorporated into spleen cells at days 3, 10, and 49. The combination of radiation and PyV infection (group A1), compared with the control (group D), reduced proliferation at all time points and to PyV infection alone (group B1) through day 20.

IFN- γ production by concanavalin A-stimulated spleen cells. Radiation alone (group C), compared with the control (group D), significantly reduced IFN- γ production at day 3 but significantly increased IFN- γ production at day 49 (Fig 1, D). PyV infection alone (group B1), compared with the control (group D), significantly increased IFN- γ production at days 10 and 20. The combination of radiation and PyV infection (group A1), compared with the control (group D), significantly decreased IFN- γ production at day 3 but increased IFN- γ production at days 10 and 20; the combination increased

IFN- γ production over the effect of radiation alone (group C) at days 10, 20, and 49, and significantly decreased IFN- γ production relative to induction caused by PyV alone (group B1) at days 3 and 20.

Effects of γ -irradiation and reactivated PyV infection on splenic cells and function

Splenic weights. PyV-infected animals that received γ -radiation at day 49 (group A1, group B1) exhibited significant decreases in spleen weights at days 54 and 59 compared with PyV-infected animals that had received no γ -radiation (group B2); no differences remained by day 69. Similarly, the doubly irradiated group (group A1) displayed decreased spleen weights at days 54 and 59 compared with those irradiated only on day 0 (group A2; Fig 2, A).

Spleen cell counts. Similar to what was observed with spleen weights, PyV-infected animals receiving

γ -radiation at day 49 (group A1, group B1) had lower spleen cell counts than other groups, especially at days 54 and 59 (Fig 2, B).

Proliferative response to concanavalin A. Irradiation at day 49 (group A1, group B1) had negligible effects on spleen cell proliferation responses to concanavalin A. However, these 2 groups of mice with detectable reactivated PyV infection at day 59 (Table II) showed the greatest depression of proliferation by spleen cells activated with concanavalin A earlier at day 54 (Fig 2, C). Interestingly, the most consistent difference between groups was observed for PyV-infected animals that received 1 dose of radiation at day 0 (group A2) compared with the nonirradiated infected animals (group B2).

IFN- γ production by concanavalin A-stimulated spleen cells. Similar to the patterns of T-cell proliferative responses, irradiation at day 49 (group A1, group B1) had modest effects on IFN- γ production by spleen cells from those animals, compared with the nonirradiated, infected group (group B2). At day 59, mice undergoing PyV reactivation in group A1 showed increased IFN- γ production, whereas animals in group B1, also undergoing PyV reactivation (Table II), did not (Fig 2, D). It appeared that IFN- γ production after virus reactivation (Fig 2, D) was less pronounced than after primary PyV infection (Fig 1, D).

DISCUSSION

Because space radiation is both constant and intermittent with solar flares, human beings will be subject to repeated doses of radiation during space flight. Under these circumstances of interplanetary space travel, latent viruses such as EBV and human PyV that are carried by virtually every human being have the potential to become reactivated, replicate, and be shed because of the radiation-induced relaxed grip of immune control. The amount of radiation that we have chosen for these studies (3 Gy) represents the estimated total dose that a space traveler would receive on a 2-year round-trip to Mars. This amount of radiation does not include that which would be experienced in a solar flare, because that exposure is unpredictable.³⁶⁻³⁸ It could be argued that the acute dose of 3 Gy does not represent a real life scenario, because it was delivered as a single exposure rather than over a period of 2 years. Because of the physical and financial constraints of the animal model, which preclude duplication of a 2-year space flight, the model is an approximation of what would be experienced on a Mars mission. It should be noted that the effects of radiation are cumulative. Previous studies have documented the toxic effects of radiation on immune cells,^{19-24,39,40} but our model adds the novel feature of simultaneous viral infection—both acute and reactivated. Thus, our model holds importance for determining the harmful effects of space radiation on immune responses and latent virus reactivation.

The measurements of immune responses used in this study were somewhat simple in nature: splenic weight,

cell count, proliferation, and IFN- γ production. Despite their simplicity, these assessments of immune structure and function yielded clear-cut effects of radiation, PyV infection, and the combination thereof. Radiation caused an acute decline in the spleen size and a decrease in the number of spleen cells, both beginning to recover by 20 to 49 days after radiation. One of the 2 functional consequences of radiation was a decrease in the ability of splenic cells to respond to activation by the T-cell agonist, concanavalin A, which had not recovered 49 days after γ -radiation. There was also an impairment of the ability of these stimulated splenic cells to secrete IFN- γ , a cytokine that is essential for the elimination of PyV infection.⁴¹ In the context of PyV infection, radiation prolonged primary virus replication in animals, and radiation of latently infected animals reactivated virus replication. Radiation further reduced the spleen weight reduction observed after acute PyV infection. Radiation reduced splenic cell proliferation in animals showing active PyV replication. The increased production of IFN- γ by splenic cells in PyV-infected animals, a normal immune response, was impaired by radiation. At day 59, more B1 mice were susceptible to undergo PyV reactivation than A1 mice, whereas IFN- γ production by splenic cells of B1 mice was significantly higher than that of A1 mice (Fig 2, D). We speculate that because repeated irradiation can render the spleen cells less capable of *de novo* protein synthesis,⁴² fewer A1 mice had PyV reactivation and they had less IFN- γ production by their splenic cells.

Although preliminary in nature, these experiments of combined γ -radiation and latent virus infection in a murine model offer a road map for the careful exploration of the potential immunosuppressive effects of some space flight conditions and their consequences that could affect human beings in interplanetary space travel. Additional experiments are needed, particularly long-term follow-up studies to observe for tumorigenesis. This model can be adapted to other forms of space radiation, most importantly the heavy metal ion radioactivity experienced in solar flare explosions.^{36,37} The critical intersection of immunosuppression, latent virus reactivation, and development of clonal malignancy needs careful evaluation, particularly as we expand our world to other planets.

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