SELECTIVE VIRAL IMMUNOSUPPRESSION OF THE GRAFT-VERSUS-HOST REACTION

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It is well known that during the acute phase of certain viral infections in man, such as measles (1) and influenza (2), a marked degree of immunological unresponsiveness exists, particularly in cell-mediated immunity. Recent studies on effects of viruses on in vitro responses of lymphocytes to antigens and mitogens strengthen the view that viruses can influence lymphocyte functions and indicate that specific viruses may affect selected classes of lymphoid cells such as T cells (3, 4), monocytes (5, 6), or B cells (7–9) (reviewed in references 10–13). These findings suggest that it might be possible, by appropriate choice of virus and experimental conditions, to harness the immunosuppressive capacity of viruses in an effort to develop a selective strategy for diminishing specific immune responses found to be clinically harmful. One such untoward response, the graft-vs.-host reaction, is reported to occur in 70% of recipients of human bone marrow transplants, and is a serious impediment to effective clinical treatment of immunodeficiency syndromes, aplastic anemias, and leukemias (14).

The present work represents an attempt to suppress graft-vs.-host reactions in an experimental model using viruses The basis for this approach derives from the observations that while resting lymphocytes are not permissive for a variety of RNA viruses, activated lymphocytes are capable of replicating viruses and may be killed by them in the process. This serves as the basis for a virus plaque assay for enumerating antigen-sensitive lymphocytes, by infecting lymphocytes stimulated by a variety of antigens or mitogens and determining the number of cells capable of producing infectious centers (15) The virus chosen for the studies is vesicular stomatitis virus (VSV),[1] a relatively nonpathogenic agent for man although mice are extremely susceptible to it. In previous in vitro studies it has been demonstrated that production of vesicular stomatitis virus occurred selectively in activated T lymphocytes rather than activated B lymphocytes in mouse and in man (3, 16).

More recent studies indicate that resting primary lymphocytes in fact may be infected by VSV, although they are unable to permit replication of the virus. When such infected cells are subsequently stimulated, the virus is somehow

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[1] *Abbreviations used in this paper* GVH, graft-vs -host; HBSS, Hanks' balanced salt solution; ^{3}HT, ^{3}H-thymidine; LN, lymph node, Mit., mitomycin; *moi*, multiplicity of infection, NDV, Newcastle disease virus; PBS, phosphate-buffered saline; PFU, plaque-forming units; VSV, vesicular stomatitis virus

activated leading to death of the stimulated cell. This has resulted, for example, in a greater than 99% suppression of thymidine incorporation by lymphocytes cultured with allogeneic mitomycin C-treated cells in mixed lymphocyte cultures. The precise requirements for establishing a latent or persistent infection by VSV in primary lymphocytes remain to be established. Nevertheless these observations suggested the possibility that histoincompatible lymphoid cells might be infected with VSV, transferred into allogeneic hosts such that activation of the infected T lymphocytes in the course of initiating a graft-vs.-host (GVH) reaction might result in the selective destruction of the responding cells. The present studies are aimed at testing the possibility that such a strategy may diminish GVH reactions in the mouse.

Materials and Methods

Mice and Virus. Young adult male A, C57L, and their F_1 hybrids, LAF_1 mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. The stock of VSV, Indiana strain, was prepared in secondary chick embryo cells as described (17). The titer was 10^9 plaque-forming units (PFU)/ml as titrated on mouse L cells

Induction of GVH Reaction. Peripheral lymph nodes (LN; i.e. axillary, mesenteric, and inguinal) were obtained from young adult A, C57L, or LAF_1 mice Cell suspensions were prepared in Hanks' balanced salt solution (HBSS) and washed. 5×10^6 LN cells were injected intravenously into LAF_1 mice 1 day after or on the day of 950 R whole body γ-irradiation (^{137}CS γ-source, Gammator M; Radiation International, Parsippany, N. J.). In some experiments the parental cells (10^7/ml) were incubated before injection with VSV ($2–2.5 \times 10^8$ PFU/ml) for 2 h at 37°C in HBSS containing 10% fetal calf serum. The cells were then washed three times with or without a subsequent addition of 0 02 ml guinea pig anti-VSV, an amount sufficient to neutralize all input virus (serum titer > 1.10,000). In other experiments, 10^8 PFU of VSV were injected directly into the recipient mice at various times after the injection of 5×10^6 parental LN cells. Preincubation of parental cells with mitomycin C (obtained through the courtesy of the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.) was performed by incubation of 10^7 cells/ml with 100 μg mitomycin (Mit.)/ml for 30 min at 37°C followed by three washings of the cells. For some experiments, it was necessary to incubate LN cells with anti-Thy 1.2 before injection. Anti-Thy 1.2 was prepared as described (18), and the conditions of the incubation were: 3.3×10^6 cells/ml, anti-Thy 1.2, 1/60; and rabbit complement (C), 1/45; 45 minutes at 37°C.

GVH Assay A modification (19) of the method of Sprent and Miller (20) was used as described. All mice received an intraperitoneal injection of 25 μCi ^{3}H-thymidine (^{3}HT; 0.36 Ci/mmol; Schwarz/Mann Div., Becton, Dickinson & Co , Orangeburg, N. Y.) 2 h before death, on days 3 and 4 after parental cell injection Mice were killed and their spleens removed. Individual spleens were quickly teased in ice-cold phosphate-buffered saline (PBS), pH 7.2, placed in tubes, and centrifuged at 1,200 rpm at 4°C for 10 min. The cells were resuspended and incubated for 2–5 min at 37°C in Tris-buffered isotonic ammonium chloride as described (19, 21) After two additional washings in PBS the cells were resuspended in 2 ml of 10% TCA and left for 18 h at 4°C. The centrifuged precipitates of each tube were dissolved in 1 ml of Nuclear-Chicago Solubilizer (NCS; Amersham/Searle Corp , Arlington Heights, Ill.) and transferred to scintillation vials containing 10 ml of a toluene base 1,4-bis[2-(5-phenyloxazolyl)]benzene-scintillation fluid. Radioactivity was determined by β-counting in a Packard series 3003 Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc , Downers Grove, Ill.).

Fate of Labeled Cells. Parental LN cells (5×10^7/ml) were labeled with ^{51}Cr (sodium chromate solution; Amersham/Searle Corp.) at 100 μCi/ml for 45 min at 37°C in HBSS containing 5% fetal calf serum. After washing the cells three times, a portion of the cell preparation was incubated with VSV as above. 10^7 cells containing $3.2–5.9 \times 10^5$ cpm were then injected intravenously into LAF_1 mice irradiated on the previous day with 950 R whole body γ-irradiation. On days 1 and 3 postinjection, recipients were exsanguinated and portions of peripheral blood, spleen, liver, lung, and peripheral LN were excised and weighed. ^{51}Cr-containing cells and tissues were counted in a

TABLE I
Effect of VSV Injection on Parental LN Proliferation in F_1 Spleen

Cells injected*	VSV injected‡	Geometric mean cpm/recipient spleen ± SE (n)§		
		Exp. 1	Exp. 2	Exp 3
Parental LN	–	55,723 × ÷ 1.05 (5)	51,779 × ÷ 1.25 (5)	11,474 × ÷ 1 21 (6)
F_1 LN	–	959 × ÷ 1.73 (5)	3,669 × ÷ 1.28 (5)	918 × ÷ 1 72 (5)
Parental LN	Day 0	2,668 × ÷ 2.10 (5)	3,362 × ÷ 1.31 (6)	887 × ÷ 1.50 (8)
Parental LN	Day 1	10,240 × ÷ 1 07 (5)		
Parental LN	Day 2	8,817 × ÷ 1.27 (5)		

* 5×10^6 LN cells injected per recipient. Parental LN in exp 1 were from strain A, in exps. 2 and 3 from strain C57L.

‡ $1\ 25 \times 10^8$ PFU of VSV injected intravenously per recipient.

§ Groups of five to eight mice were assayed 3 days after LN cell injection They were injected intraperitoneally with 25 μCi ^{3}HT and killed 2 h later.

Nuclear-Chicago Ultrascaler II γ-counter without prior solubilization (Nuclear-Chicago Corp , Des Plaines, Ill.)

Results

Inhibition of GVH Reactivity of Parental Cells by VSV. Irradiated LAF_1 mice were injected intravenously with 5×10^6 syngeneic or parental LN cells. 3 days after cell injection, a marked DNA synthetic response was observed in the spleens of mice injected with parental cells, much higher than after injection of syngeneic cells (Table I). Previous studies have shown, in fact, that syngeneic cells do not usually cause a significant increase of ^{3}HT incorporation over that observed in uninjected control mice and that treatment of parental LN cells with anti-Thy 1.2 plus C brings the level of ^{3}HT incorporation down to that seen with syngeneic cells (19). Treatment of parental cells with Mit., that is known to inhibit DNA synthesis (22), also prevents the splenic proliferative response (19).

When recipients of 5×10^6 A (exp. 1, Table I) or C57L (exps. 2 and 3, Table I) were simultaneously injected with 1.25×10^8 PFU of VSV intravenously, the resulting GVH activity as measured by ^{3}HT incorporation on day 3 was virtually completely inhibited. Recipients injected with VSV 1 or 2 days after parental cell injection still showed a much reduced response, even though the assay was performed on day 3. Since previous studies clearly showed that a detectable proliferative response is present on day 2 (19) this indicates that the virus eradicated an already initiated response. Thus, injection of VSV inhibits the GVH reaction as detected by the spleen assay.

Because preinfection of lymphocytes was able to block subsequent proliferation in mixed lymphocyte cultures (reference 13 and unpublished observations), we attempted to block the GVH reaction by infecting the parental LN cells in vitro before transferring them to F_1 recipients. The cells were infected at a multiplicity of infection (*moi*) of 20–25 for 2 h at 37°C and washed three times before injection. Such preinfected cells failed to generate GVH parental cell

TABLE II
Effect of VSV Preinfection on Parental LN Proliferation in F_1 Spleen

Cells infected*	VSV incubation‡	Treatment with anti-VSV§	Geometric mean cpm per spleen SE (n)			
			Exp 4	Exp 5	Exp 6	Exp 7
Parental LN	–	–	13,091 ×÷ 1 45 (3)	55,723 ×÷ 1 05 (5)	51,779 ×÷ 1 25 (5)	71,979 ×÷ 1 24 (5)
F_1 LN	–	–	7,642 ×÷ 1 45 (3)	959 ×÷ 1 73 (5)	3,669 ×÷ 1 28 (5)	447 ×÷ 1 91 (6)
Parental LN	+	–	1,004 ×÷ 1 64 (4)	2,554 ×÷ 1 34 (5)	7,657 ×÷ 1 31 (5)	
Parental LN	+	+	3,076 ×÷ 1 72 (6)		8,639 ×÷ 1 35 (6)	8,418 ×÷ 1 32 (5)
F_1 bone marrow	–	–	16,964 ×÷ 1 20 (9)	35,426 ×÷ 1 31 (6)		
F_1 bone marrow	+	–	14,434 ×÷ 1 10 (6)	28,660 ×÷ 1 09 (4)		

* 5×10^6 LN cells or 10^7 bone marrow cells injected per recipient Spleens assayed on day 3 (exps 4, 5, and 6) or day 4 (exp 7) after cell injection

‡ Cells were incubated with VSV (moi 20–25) for 2 h at 37°C LN cells but not bone marrow cells were washed three times before injection

§ After VSV incubation and one wash cells were incubated at 37°C for 1 h with 1/200 guinea pig anti-VSV They were then washed twice and injected into recipients at a dose of 5×10^6 LN cells per recipient

proliferative activity (Table II). The degree of inhibition in exps. 4, 5, and 6 actually amounted to 92, 96, and 85%, respectively. It could be argued that free virus particles were in the injected cell suspensions despite the extensive washing of the cells and that it was this free virus rather than lymphocyte-associated virus that was inhibiting the GVH. To eliminate free infectious virus particles in the inoculum the cells were incubated for an additional h at 4°C with 1/200 guinea pig anti-VSV serum, a concentration sufficient to neutralize all input virus particles. After cells so treated were injected into recipients, the GVH was still markedly suppressed (76% in exp. 4, 83% in exp. 6, and 89% in exp. 7).

Specificity of VSV Immunosuppression. It has been shown that VSV can inhibit lymphocyte activation induced in vitro by concanavalin A or by the mixed lymphocyte reaction, but not the one induced by lipopolysaccharide (3), suggesting a predilection of VSV for T cells. To study further this apparent inability of VSV to act on non-T cells, F_1 bone marrow cells (10^7 per recipient), with or without preincubation with VSV, were injected into lethally irradiated syngeneic recipients and the degree of proliferation in the spleen measured 3 days after cell injection. In neither exp. 4 nor exp. 5 (Table II) was a significant reduction in proliferation seen after incubation with the virus (Student's t test: $0.1 < P < 0.2$ in exp. 4 and $0.5 < P < 0.6$ in exp. 5).

It was found previously that in GVH reactions intravenous injection of a mixture of mitomycin C-treated parental LN and anti-Thy 1.2-treated F_1 LN gave a good proliferative response while neither cell type alone synthesized DNA in appreciable amounts (19). This phenomenon is again demonstrated in Table III. While neither 5×10^6 syngeneic LN, whether anti-Thy 1.2-treated or not, nor 5×10^6 Mit.-treated parental LN injected alone resulted in a high incorporation of ^{3}HT by day 3, the simultaneous injection of both cell types gave a much higher response (approximately 35 and 59% of the response seen with untreated parental cells). As was also previously determined, the LAF_1 non-T cells made up the bulk of this syngeneic LN proliferation (19). It should be noted

TABLE III

Relative Insensitivity to VSV of Syngeneic B-Cell Proliferation in GVH

Cells Injected*	Geometric mean cpm/recipient spleen SE (n)‡	
	Exp 8	Exp. 9
F_1 LN	1,425 ×÷ 1 36 (5)	918 ×÷ 1 74 (6)
Parental Mit LN§	474 ×÷ 1.26 (5)	689 ×÷ 1.29 (6)
Anti-Thy 1 2-treated‖ parental LN	916 ×÷ 1.26 (5)	626 ×÷ 1.20 (6)
Parental LN	24,158 ×÷ 1.45 (5)	11,474 ×÷ 1.20 (6)
Parental VSV-LN¶	721 ×÷ 1 59 (6)	549 ×÷ 1.59 (6)
Parental Mit. LN + anti-Thy 1.2-treated F_1 LN	9,325 ×÷ 1.26 (7)	6,533 ×÷ 1 20 (6)
Parental Mit. LN + anti-Thy 1.2-treated F_1 LN incubated with VSV	4,379 ×÷ 1 95 (5)	3,812 ×÷ 1.38 (6)
Parental Mit. LN incubated with VSV + anti-Thy 1.2-treated F_1 LN	ND	867 ×÷ 1.26 (6)

* 5 × 10^6 LN cells injected per recipient Where mixtures of different cell populations were concerned, 5 × 10^6 LN cells of each cell type were injected.

‡ Mice were assayed on day 3. They were killed 2 h after an intraperitoneal injection of 25 μCi ^{3}HT

§ Mit LN, lymph node cells treated with 100 μg/ml Mit. for 30 min at 37°C.

‖ LN cells were treated with 1/60 anti-Thy 1.2 and 1/45 rabbit C for 45 min at 37°C at a final concentration of 3.3 × 10^6 cells/ml

¶ Cells incubated for 2 h at 37°C at a concentration of 10^7/ml with VSV at a concentration of 2.5 × 10^8 PFU/ml

that incubation of the anti-Thy 1.2-treated F_1 LN cells with VSV followed by washing still allowed them to be recruited by Mit.-treated parental cells (exps. 8 and 9, Table III) although the ^{3}HT incorporation was about half the amount seen without VSV incubation of the F_1 cells. This suggested that the non-T elements were relatively insensitive to the action of VSV, and certainly much less sensitive than the parental T cells since their activity was totally abolished by the same amount of virus (3–4% of the response remaining). It was of additional interest to note that Mit.-treated parental LN cells which had been preincubated with VSV were no longer capable of recruiting F_1 B cells into proliferation. It appears that the VSV incubation has an even more drastic inhibitory effect on the T cells than Mit. treatment.

Effect of Preincubation with VSV on the Subsequent Homing of Parental LN Cells in F_1 Hosts. A possible criticism of the conclusion that VSV suppresses GVH is that the virus might alter the homing pattern and organ distribution of parental LN cells, thereby reducing the number of parental cells in the spleens of recipients (23). Thus, the low ^{3}HT incorporation in the recipients' spleens could be due to a failure of parental LN to home to spleen instead of an actual destruction of reactive allogeneic T blasts by VSV. To differentiate between these possibilities, experiments were performed to examine the effect, if any, of preincubation with VSV on the fate of injected cells. Parental LN cells were

TABLE IV

*Effect of VSV Incubation on the Organ Distribution of ^{51}Cr-Labeled Parental LN Cells**

VSV treatment‡	Day after injection	% of injected cpm/100 MG Tissue				
		Spleen	LN	Liver	Lung	Blood§
−	1	31.04 ± 3.2‖	2.50 ± 1.1	3.12 ± 0.3	2.78 ± 0.6	5.79 ± 0.3
+	1	42.74 ± 1.3	1.53 ± 0.6	6.60 ± 0.8	1.62 ± 0.2	11.23 ± 0.8
−	3	32.25 ± 2.6	1.97 ± 0.7	4.04 ± 0.5	1.98 ± 0.5	4.28 ± 0.8
+	3	38.42 ± 3.3	3.18 ± 0.7	5.63 ± 0.5	0.81 ± 0.2	6.37 ± 0.6

* Intravenously injected 3.2–5.9 × 10^5 cpm (10^7 cells) into 950 R γ-irradiated LAF_1 mice (four to five per group).

‡ Cells incubated for 2 h at 37°C at a concentration of 10^7/ml with VSV at a concentration of 2.5 × 10^8 PFU/ml.

§ cpm expressed as % of injected cpm/10 ml blood

‖ Mean ± SE.

labeled with ^{51}Cr and, after washing, a portion of these labeled cells was further incubated with VSV (*moi* 25) as usual. After washing, the cells were intravenously injected and organs were removed 1 and 3 days later. VSV pretreatment did not cause any reduction in the amount of γ-radioactivity present in the spleen at either time after cell injection (Table IV). It should be noted that a decrease of as much as 12% (24), which is the percentage of parental cells estimated to respond against F_1 hybrids in the rat and which would have been destroyed by VSV which infects responding cells, would not be detected in this type of experiment.

Discussion

The present results demonstrate that VSV infection under appropriate conditions is capable of inhibiting the proliferative phase of the GVH response in mice. Further, they confirm previous observations made in vitro (3, 16) that VSV affects primarily T lymphocytes, and indicate that cells preinfected in vitro are killed by the virus when they are activated against alloantigens in vivo. The relative resistance of non-T-cell responses and of hematopoietic cells is borne out by the lack of inhibitory effect of VSV on their proliferation. The B-cell nature of the resistant recruited cell was established in previous work (19). It is interesting to note, however, that the inhibition of the T-cell response by VSV in the present studies included an abolition of the recruiting ability of these cells as well. This might have been expected in view of the fact that VSV rapidly inhibits host cell macromolecular synthesis upon activation (25–27) and this would prevent production of a factor responsible for the recruitment of B cells. No such inhibition is seen when T cells are inhibited merely from proliferating by mitomycin treatment and can also induce delayed hypersensitivity reactions (28). In vivo injection of VSV or in vitro treatment by VSV before injection of T cells appears to lead to a total abolition of both T-cell proliferation and T-cell-induced responses.

It is of obvious interest to attempt to apply this information to the prevention or treatment of secondary disease in vivo caused by allogeneic or semiallogeneic

spleen or bone marrow transplants in irradiated hosts. While preinfection with VSV can abrogate the reactivity of T cells, the obvious consequence in the immunologically compromised host would be a viremia and possibly viral disease. Thus development of this strategy for treatment of humans would require protection of the host against the consequences of virus infection. Two possibilities for avoiding this complication are envisioned. First, the host could be protected against VSV by treatment with anti-VSV serum. Preliminary findings (to be published) suggest that suppression of spleen-induced secondary disease can indeed be effected by prior incubation of the parental spleen cells with VSV, and that the recipients survive if they are protected from disseminated virus infection by treatment with anti-VSV antibody. The present results with anti-VSV antibody incubation before injection of VSV-infected cells also suggest that virus had been adsorbed and penetrated into the T cells and could no longer be inhibited from exercising its lytic effect on these cells by anti-VSV. A second possibility would be to employ temperature-sensitive mutants of the virus which would be capable of killing the T cells at a nonpermissive temperature (37°C) without producing infectious virus, as appears to be the case for certain tissue culture cells (29). Preliminary data indicate this to be possible with at least two ts mutants of VSV in human lymphocytes (to be published).

In in vitro studies on suppression of mixed lymphocyte cultures it has become clear that not all preparations of VSV are effective in establishing latent or persistent infections in lymphocytes. Preliminary evidence suggests that defective interfering virus particles may play an important role. It will be important, therefore, to establish the optimal conditions for infecting normal lymphocytes to achieve maximal selective immunosuppression upon later stimulation.

Woodruff and Woodruff found that Newcastle disease virus (NDV), another T-cell specific virus (12), inhibited skin graft rejection in mice (30) but the effect was only transient in spite of daily injections of virus. These authors suggested that the effect might be caused by a change in surface structure of the lymphocytes which temporarily altered their normal migration pattern in the host (23, 31). It is also possible that during the course of skin graft rejection the recipients produced antibodies to the virus and neutralized its effects.[2] In the present studies, VSV-infected cells showed an undiminished tendency to accumulate in the spleens of host mice although their proliferation was inhibited. Similarly McFarland (4) has found that infection of mice with measles virus selectively inhibits the T-cell helper response, thus reinforcing the impression that selective T-cell responses may be abrogated by certain viral infections in vivo.

There remain a number of problems to be explored in this model. It will be important to establish the degree to which B-cell function is restored in the recipients, particularly in terms of thymus-dependent and thymus-independent antibody responses. Similarly, it will be of interest to ascertain the degree to

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[2] A recent paper by McGregor et al (*J Exp Med.* 144:627, 1976) has appeared in which it is reported that in vitro infection of sensitized thoracic duct lymphocytes with NDV blocked their ability to transfer cellular resistance to *Listeria monocytogenes*, and delayed-type hypersensitivity to soluble Listeria antigens While NDV, in contrast to VSV, may act both by altering the migration pattern of the cells, it is likely that the principal mechanism of immunosuppression of the preinfected cells is similar to that described here

which T-cell reconstitution of the allogeneic spleen occurs, and whether any helper activity, proliferating activity, or cytotoxic T-lymphocyte activity can be developed to unrelated antigens. Finally, the duration of the immunosuppression of GVH reactions in animals protected against the effects of infectious virus will be important to determine, in order to learn whether this strategy is effective only against acute GVH reactions or may also diminish the chronic manifestations as well.

Summary

Graft-vs.-host (GVH) reactivity of parental lymph node (LN) cells was assayed by measurements of ^{3}H-thymidine incorporation in vivo in spleens of irradiated F_1 recipients. Preincubation of parental LN cells with vesicular stomatitis virus (VSV) for 2 h at 37°C followed by washing resulted in an 85–90% reduction in splenic radioactivity, as did injection of VSV on days 0–2 after recipients received untreated parental LN cells. In contrast, ^{3}H-thymidine incorporation in the spleens or irradiated F_1 hosts was not affected by VSV when F_1 bone marrow cells were incubated with the virus. In addition, preincubation of F_1 B cells with VSV still allowed these syngeneic B cells to be recruited into proliferation by mitomycin-treated parental LN cells. The inhibitory effect of VSV, thus, seems to be specific for T-cell proliferation. These observations suggest that viral immunosuppression might be capable of being developed into a useful strategy for selective deletion of lymphocytes capable of reacting against histocompatibility antigens and initiating GVH reactions.

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