

Replication of Vesicular Stomatitis Virus in Murine Spleen Cells: Enrichment of the Virus-Replicating Lymphocytes and Analysis of Replication Restriction

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Vesicular stomatitis virus (Indiana strain) will only grow in T lymphocytes which have been stimulated to undergo cell division. Evidence is presented that a considerable enrichment of the vesicular stomatitis virus-replicating T cells may be accomplished in the mouse spleen by passing the spleen cells over glass wool columns. By using this procedure experiments were performed to study the nature of the block in vesicular stomatitis virus replication in unstimulated (nonpermissive) versus mitogen-stimulated (permissive) splenic T cells. The results show that, as is the case in permissive T-cell lines, stimulated normal T cells allow the synthesis of the 42S virion ribonucleic acid.

Unstimulated, resting lymphocytes are nonpermissive for viral replication (2). Upon activation by antigens or mitogens, lymphocytes quickly gain the capacity to replicate a variety of viruses (2, 8). Of particular interest have been the studies of Bloom and his colleagues on the replication of vesicular stomatitis virus (VSV) in activated lymphocytes (2, 3, 7-10). The express purpose of those studies was to try to develop a method which would allow the enumeration of antigen-activated T cells in a fashion similar to that used for enumerating antibody-producing B cells (2). Although problems have arisen about the utility of this system for the detection of antigen-reactive T cells (4, 5), the use of VSV to study the permissive versus nonpermissive state of activated versus nonactivated T cells remains an interesting topic. In this regard the use of VSV and fresh normal lymphocytes presents additional problems. The principal difficulty is that the spleen cells which replicate VSV range between 0.1 to 6% of the total spleen cell population. As has been pointed out, this is not a sufficiently large number of lymphocytes to permit the convenient study of the cellular parameters involved in the acquisition of permissiveness (7, 8). Studies by Nowakowski et al. (7) several years ago showed that in a nonpermissive lymphoblastoid line (Raji) the restriction in viral replication seemed to involve a step required for the replication of the 42S virion ribonucleic acid (RNA).

In this communication we present evidence indicating that it is possible to select a subpopulation of spleen cells in which up to 20% of the cells will replicate VSV after mitogen stimulation. Furthermore, as with the experiments of Nowakowski et al. (7), restriction of replication

in normal T cells involves a step required for the replication of 42S virion RNA.

MATERIALS AND METHODS

Mice. C57BL/6 males, 6 to 10 weeks of age, were used throughout these studies.

Virus. VSV Indiana strain was purified as described previously (1).

Cell preparation and isolation. Spleen cells were isolated by previously described methods. The culture medium used throughout the experiments was RPMI 1640 supplemented with penicillin-streptomycin and L-glutamine (11). Isolation of spleen cell subpopulations based on adherence to glass wool was carried out as reported by Webb and Jamieson (11).

Mitogens. Phytohemmagglutinin (PHA; HA 17, Burroughs Wellcome, Research Triangle Park, N.C.) was stored in RPMI 1640 at -20°C . Concanavalin A (ConA; Miles Laboratories, Kankakee, Ill.) was stored at -20°C as a powder and reconstituted with RPMI 1640 before use. Bacterial lipopolysaccharide (LPS; *E. coli* strain B, Difco Laboratories, Chicago, Ill.) was stored as a powder at -20°C and reconstituted in RPMI 1640 before use.

Infectious centers assay. The infectious centers assay used was essentially that described by Bloom and his colleagues (2, 3). Briefly, cells at a concentration of $10^7/\text{ml}$ were exposed to VSV at a multiplicity of infection of 20 plaque-forming units per cell at 37°C with continuous rocking. After 2 h the cultures were treated with anti-VSV antiserum (kindly provided by R. A. Lazzarini, National Institutes of Health, Bethesda, Md.) at a dilution which had previously been determined to neutralize the virus. The infected cells were washed three times in minimum essential medium, and duplicate samples were added in varying dilutions to a medium containing 1% agar and plated over a layer of indicator L cells. Viral plaques were scored after 48 h.

Radiolabeling of VSV-specific RNA. Cells were infected at an multiplicity of infection of 20 plaque-

forming units per cell, with actinomycin D (5 µg/ml) also added. After 3 h [³H]uridine was added (20 µCi/ml; specific activity, 28 Ci/mmol), and the cells were incubated for an additional 20 h. At the end of this period, the cells were prepared for RNA extraction.

Isolation and analyses of VSV-specific RNA. The [³H]uridine-labeled cells were centrifuged at 35,000 rpm for 30 min with a Beckman 70 Ti rotor. After a second wash, the cell pellet was resuspended in reticulocyte standard buffer (RSB) and placed on ice for 10 to 15 min. Nonidet P-40 was then added to a final concentration of 1%, and the cell suspension was allowed to sit for an additional 10 min. The suspension was then centrifuged at low speed for 2 min, and the supernatant was collected. Sodium dodecyl sulfate was added at a final concentration of 1%, and the suspension was extracted with an equal volume of phenol (saturated with 0.01 M Tris-0.001 M EDTA buffer [pH 8.0]). The suspension was thoroughly mixed and incubated at 60°C for 2 min. The mixing and 60°C incubation step was repeated three times. The suspension was centrifuged, and the aqueous phase was collected. The phenol phase was mixed with an equal volume of RSB, and the suspension was treated as above. The aqueous phase extract was made up to 0.4 M NaCl, and 2 volumes of ethanol and 100 µg of carrier transfer RNA were added. The precipitate was collected after 24 h, and the remaining ethanol was removed by lyophilization. The dried precipitate was resuspended in sterile distilled water containing 1% sodium dodecyl sulfate and layered on a 15 to 30% sodium dodecyl sulfate sucrose gradient. The gradient was centrifuged for 12 h at 20,000 rpm in a SW40 rotor at 20°C. Aliquots (200 µl) were collected from the bottom of the tubes, precipitated with ice-cold 5% trichloroacetic acid, and then assayed for radioactivity by scintillation spectroscopy.

RESULTS

Effects of PHA and LPS on virus replication in whole spleens and splenic subpopulations. Our early experiments were directed toward developing a way to isolate subpopulations of lymphocytes which replicate virus. In the experiment shown in Table 1, spleen cells or subpopulations of non-glass-adherent (NAL) or glass-adherent lymphocytes (GAL) were stimulated with 2.5 µg PHA or LPS per ml (0.4 µg/ml). These doses were chosen because they had been shown previously to be optimal for stimulation of deoxyribonucleic acid synthesis (11). After 24 h the various cultures were mixed with VSV and checked for viral replication by the infectious center assay. As can be seen in Table 1, LPS did not activate any of the cell populations to become permissive although both NAL and GAL contain 20 and 60% B cells, respectively (11). In contrast, PHA activated spleen cells and the subpopulations in such a way so that they became permissive. This is in agreement with the reports of Bloom and his co-workers (2, 3, 7-10) as well as other investigators (4, 5). Of particular interest is that the NAL

population of spleen cells (80% T cells, 15% B cells; 2 to 5% macrophages [11]) contained a much larger proportion of permissive cells than the GAL population. Thus, by the use of a simple separation procedure, a considerable enrichment of virus-replicating cells can be achieved. To further confirm that this subpopulation of spleen cells contained increased numbers of permissive lymphocytes, a dose-response to PHA and ConA was carried out. As depicted in Table 2, increasing doses of PHA or ConA yielded an increased percentage of infectious centers. The number of infectious centers was two to three times greater than the maximum observed by us in whole spleens or as reported by others (2, 3, 7-10).

Measurement of viral RNA in infected NAL stimulated with PHA. Nowakowski et

TABLE 1. *Effect of PHA and LPS on viral replication in separated spleen cell populations*

Cells	Treatment	Plaques/ 10 ⁶ cells ^a	% Infectious centers
Whole spleen		100	0.01
Whole spleen	PHA (2.5 µg/ml)	30,000	3.0
Whole spleen	LPS (0.4 µg/ml)	300	0.03
NAL		300	0.03
NAL	PHA	200,000	20.0
NAL	LPS	0	0
GAL		300	0.03
GAL	PHA	15,000	1.5
GAL	LPS	0	0
NAL + GAL		32,000	3.2
NAL + GAL	PHA	200,000	20.0
NAL + GAL	LPS	0	0

^a The numbers represent the average of duplicate determinations.

TABLE 2. *Dose-response to PHA and ConA with NAL isolated from whole spleens*

Mitogen	Dose (µg/ ml)	Plaques/10 ⁶	% Infectious centers
PHA	0	100 ^a	0.01
	0.5	36,000	3.6
	1.0	56,000	5.6
	2.5	100,000	10.0
	5.0	96,000	9.6
ConA	0	20,000	2.0
	0.5	14,000	1.4
	1.0	16,000	1.0
	2.0	40,000	4.2
	4.0	100,000	10.0
	6.0	150,000	15.0

^a These numbers represent the average of duplicate determinations.

al. (7) had previously demonstrated that the difference between permissive and nonpermissive lymphoblastoid cell lines was related to the ability of the permissive cells to allow replication of the 42S virion RNA. The preceding experiments had demonstrated a sufficient degree of enrichment of virus-replicating cells to allow a similar experiment to be performed. Accordingly, NAL were cultured with or without PHA (2.5 $\mu\text{g}/\text{ml}$) for 18 h and then infected with virus (multiplicity of infection, 20). During the infection actinomycin D (5 $\mu\text{g}/\text{ml}$) was present to inhibit cellular RNA synthesis. After 3 h of incubation at 37°C, [^3H]uridine was added (20 mCi/ml) for 21 h. At the end of the incubation period, the cells were collected and washed, and the RNA was extracted as outlined in Materials and Methods. The RNA was analyzed by sodium dodecyl sulfate-sucrose density gradient centrifugation. A typical result is depicted in Fig. 1. In unstimulated, nonadherent cells there was 18S RNA present but little 42S RNA, whereas in PHA-stimulated cells there was clearly a much larger amount of 42S RNA as well as 18S RNA. This is in agreement with the results obtained by Nowakowski et al. (7) with human lymphoblastoid cell lines, except that no 26S virus-specific RNA species were detected. To confirm that the 42S RNA was indeed the genomic RNA

of VSV, the RNA collected from the gradient was hybridized with VSV 12 to 18S messenger RNAs, representing half of the genome length, obtained from *in vitro* transcription reaction (6). The data not shown indicated that increasing amounts of VSV messenger RNAs hybridized with the genomic RNA and, as expected, protected 50% of the radioactivity from ribonuclease digestion when the hybrid was treated with ribonuclease T₂. Similarly, the 18S RNA (Fig. 1) hybridized to the VSV genome RNA, indicating that it contained VSV messenger RNA species (data not shown).

DISCUSSION

The data presented in this report show that it is possible to isolate the majority of the VSV-replicating cell population of the spleen by relatively simple separation technology. This enrichment is sufficient to allow the investigation of questions relating to replication restriction of VSV in normal lymphoid cells after activation by polyclonal mitogens. As illustrated in Table 1, the observed number of cells replicating VSV in whole spleen cell cultures is an order of magnitude lower than that observed when T cells are enriched by passage over glass wool and cultured with mitogen and virus. In fact, without this enrichment it is not possible to measure VSV-specific RNA synthesis in whole spleen cell cultures (data not shown). The enrichment procedure used here had already been successfully employed to study aspects of T-lymphocyte regulation because virtually all of the PHA-responsive T cells are not glass adherent (11). This suggested to us that it might be possible to address the question of replication restriction of VSV in the enriched population of normal T lymphocytes. The data shown in Tables 1 and 2 reflect the fact that enrichment for T-cell-specific, mitogen-responsive lymphocytes also enriches for VSV-replicating cells. When these same lymphocytes are exposed to a B-cell mitogen, LPS, no VSV replication occurs since T cells are not activated. In Table 2, with varying doses of T-cell mitogens it may be seen that as the mitogen dose increases the number of cells replicating VSV increases. This is to be expected since at the doses of mitogen used, increasing levels of lymphocyte blastogenesis occur (11). The results observed from the sucrose density gradients show that in the absence of lymphocyte activation by PHA VSV is unable to synthesize the 42S genomic RNA, although it may synthesize primary transcripts sedimented at 18S presumably mediated by the virion-associated transcriptase (Fig. 1). This implies that the host lymphocyte, after activation by mitogens, provides the necessary elements needed by the

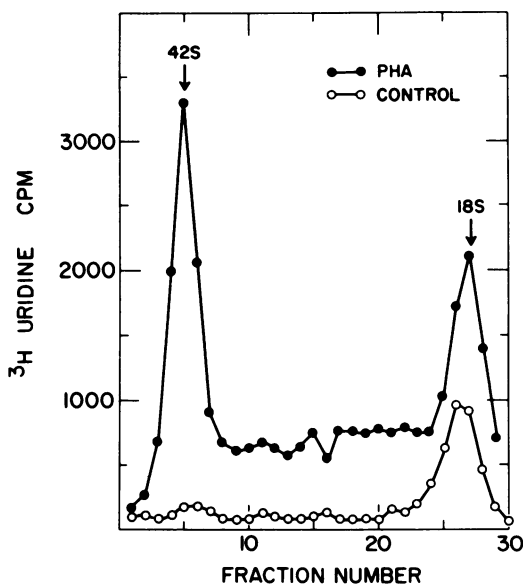


FIG. 1. Analysis by sucrose density gradients of viral RNA isolated from NAL stimulated with PHA. NAL isolated from whole spleens were treated with PHA and exposed to VSV as outlined in the text. The RNA was labeled with [^3H]uridine and analyzed by sucrose density gradient centrifugation as described in the text.

virus for complete genome replication. It remains to be determined what this element or elements are and at precisely what stage of viral replication they function.

These results confirm the work of Nowakowski et al. (7) with lymphoblastoid cell lines which were either restricted or permissive for VSV replication. Data from these workers showed that a restricted line also failed to allow replication of genomic RNA, suggesting that for this line (Raji) the restriction may be similar or the same as that observed with normal cells.

It will now be possible to compare levels of restriction among lymphoblastoid lines and normal cells to determine the mechanisms which control VSV replication.

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