Clearance of a Persistent Paramyxovirus Infection Is Mediated by Cellular Immune Responses but Not by Serum-Neutralizing Antibody

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Infection of the lungs of immunodeficient mice with the paramyxovirus simian virus 5 (SV5) was prolonged compared with the time course of infection in immunocompetent mice. Although there was a significant increase in both viral RNA and proteins, little infectious virus was produced. Adoptive transfer of immune lymphocytes (isolated from the spleens of mice previously infected with SV5) but not of nonimmune lymphocytes increased the speed of clearance of virus from the lungs of immunodeficient mice. In contrast, passive transfer of a pool of neutralizing monoclonal antibodies specific for the HN and F glycoproteins of SV5 did not have a significant effect on the speed of clearance of virus. Furthermore, no significant increase in the rate of virus clearance was observed upon adoptive transfer of purified immune B lymphocytes to SV5-infected immunodeficient mice despite production by the mice of high titers of neutralizing antibodies. Evidence is presented that CD8⁺ effector cells are primarily responsible for the clearance observed. The general significance of these results with respect to immune clearance of persistent virus infections is discussed.

Paramyxoviruses can cause persistent infections *in vitro* and *in vivo*; in such infections, only small amounts of infectious virus may be produced but virus RNA and proteins can be detected over prolonged periods of time. *In vivo*, such infections may have a number of important consequences for both the host and the virus (R. E. Randall and W. C. Russell, in D. W. Kingbury, ed., *The Paramyxoviruses*, in press). Indeed, a number of chronic human diseases have been shown to be either caused by persistent paramyxovirus infections (e.g., measles and subacute sclerosing panencephalitis; 25) or linked with such infections (e.g., Paget's bone disease, autoimmune chronic active hepatitis, and multiple sclerosis; reviewed by Randall and Russell [in press]). Nevertheless, it appears that before the establishment of a persistent infection *in vivo*, the infected individual often survives the acute infection in a normal fashion. There are therefore two major questions that need to be addressed in such situations: (i) what are the molecular mechanisms involved in the establishment of persistent paramyxovirus infections, and (ii) why does the immune system fail to clear the persistent infection when it copes successfully with the acute infection.

An acute virus infection is normally controlled through the induction and interaction of specific antibody and cell-mediated immune responses. Antibodies are primarily concerned with the inactivation, or neutralization, of free virus, whereas cytotoxic T lymphocytes (CTLs) recognize and kill virus-infected cells and thus prevent or reduce the release of progeny virus. T lymphocytes may also mediate antiviral activity through the release of lymphokines, such as gamma interferon. T lymphocytes recognize virus antigens in association with either class I or class II major histocompatibility complex (MHC) antigens. Although in most virus infections class I-restricted CTL responses are the predominant response, for some viruses it appears that class II-restricted

CTLs may also be important. Class I- and class II-restricted T cells can be distinguished by monoclonal antibodies to cell surface antigens, those recognizing class I MHC antigens having a CD8⁺ CD4⁻ phenotype and those recognizing class II MHC antigens having a CD4⁺ CD8⁻ phenotype (reviewed in reference 17a).

Previous work has shown that CTLs play a major role in clearing both orthomyxovirus and paramyxovirus infections from infected mouse lungs (5, 14, 24, 27). There have also been numerous reports demonstrating that antibodies to the surface glycoproteins of these viruses are protective *in vivo* (8, 13, 21, 23, 26, 28). In these cases, it is generally agreed that the protection observed is mainly due to the ability of these antibodies to neutralize virus infectivity. We have previously shown that a prototype paramyxovirus, simian virus 5 (SV5), is capable of infecting mouse lungs and that immunization with either internal or external structural proteins as solid matrix-antibody-antigen complexes increases the speed of clearance of the virus from the lungs. However, in these studies there was no correlation between the speed of clearance and the level of serum-neutralizing antibodies in immunized mice (20), suggesting that cell-mediated immunity was responsible for virus clearance. During a time course of SV5 infection of immunocompetent mice, increasing amounts of virus proteins and nucleic acids can be detected, by Western immunoblot analysis and *in situ* hybridization, in their lungs until 3 days postinfection (20). Thereafter, the amount of virus within the lungs remains relatively constant until 7 days postinfection, after which time there is a rapid decrease. However, we wished to develop this mouse model system so as to be able to further dissect the immune mechanisms responsible for virus clearance. We were also interested in determining whether such a system could be developed for use as a model system for paramyxovirus persistence. Since it had been reported that respiratory syncytial virus causes a persistent infection in mice made immunodeficient by X irradiation (5), we decided to examine the course of infection of SV5 in immunodefi-

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cient mice. In this report, we demonstrate that SV5 also establishes a persistent infection in the lungs of immunodeficient mice and show that whereas cell-mediated immune responses clear this infection, neutralizing antibodies do not.

MATERIALS AND METHODS

Cells and virus. BHK and Vero cells (Flow Laboratories) were grown as monolayers in 96-well microtiter plates, 75-cm² tissue culture flasks, or rotating 80-oz (ca. 2.4-liter) Winchester bottles in Dulbecco modified Eagle tissue culture medium containing 10% newborn calf serum. P815, EL4, and RIE cells and suspension HeLa cells were grown in suspension growth media. A human isolate of SV5 (LN; 9) was grown and titrated under appropriate conditions in Vero or BHK cells, using medium containing 2% calf serum.

X irradiation of mice. BALB/c mice (6 to 8 weeks old) were exposed to 5 Gy of whole-body X irradiation (⁶⁰Co source) before infection with SV5. Immediately after the mice were X irradiated, their splenocytes failed to respond to the B-cell mitogen lipopolysaccharide (LPS) and the T-cell mitogen concanavalin A (ConA). By 10 days after irradiation, the spleens had atrophied to such an extent that only 2×10^6 to 4×10^6 splenocytes could be isolated per spleen, as opposed to 1×10^8 to 2×10^8 from a normal spleen. The few splenocytes isolated from X-irradiated mice at this time showed some activation by ConA but none by LPS. X-irradiated mice do, however, begin to recover the ability to mount an immune response 2 to 3 weeks after irradiation (1), and 10 weeks after irradiation the spleens had increased in size such that 2×10^7 to 4×10^7 cells could be isolated per spleen and the splenocytes proliferated in the presence of both LPS and ConA (unpublished observations).

Infection of mice. While anesthetized with ether, mice were infected by inhalation of 5×10^6 to 10×10^6 PFU of the LN strain of SV5 in 90 μ l of culture medium. At various times after infection, the mice were sacrificed and the lungs were removed, weighed, and frozen at -70°C until required.

Passive transfer of monoclonal antibodies. A pool of monoclonal antibodies (SV5-HN-1c, -3a, -4a, -4c, -4f, -5b, -5c, -5d, and -5e and SV5-F-1a; 19) to the HN and F glycoproteins of SV5 was made. The equivalent of 20 μ l of each anti-HN antibody and 80 μ l of the anti-F antibody, as ascitic fluid, was passively transferred to mice by intraperitoneal inoculation. Mice had neutralizing titers of 1/500 to 1/2,000 at 1 day or 10 days posttransfer.

Adoptive transfer of different lymphocyte populations to immunodeficient mice. BALB/c (*H-2^d*) mice were immunized twice by infection with SV5 as described above, with a gap of 4 to 8 weeks between infections. Spleen cells were isolated from immune mice 4 to 6 weeks after the second infection by standard methods. Then 2×10^7 to 4×10^7 unselected or selected (see below) lymphocytes were adoptively transferred by intraperitoneal inoculation in phosphate-buffered saline (PBS). B lymphocytes were purified or depleted from certain lymphocyte preparations (as indicated) by using immunoaffinity plates as previously described (17). Briefly, monolayers of fixed and killed suspensions of *Staphylococcus aureus* Cowan A were prepared and stored dry for up to 1 week at room temperature. Anti-mouse immunoglobulin was bound to these plates by incubating the plates with a 1/20 dilution of sheep anti-mouse immunoglobulin (SAPU, Lanarkshire, Scotland) in PBS at 4°C for 4 to 6 h with continual rocking. Adherent cells were first removed by incubating 5×10^7 to 7×10^7 splenocytes with immunoabsorbent monolayers at 37°C for 1 h in tissue culture

medium. The nonadherent cells were then transferred to anti-mouse immunoglobulin plates and incubated at 4°C for 1 h. The nonbound cells were removed by gently rocking the plates, harvesting the medium, and carefully dropping the medium back onto the plates a number of times. If purified B-cell populations were required, the plates were washed three more times with ice-cold PBS, culture medium was added, and the plates were incubated at 37°C for 2 to 4 h. Bound lymphocytes were removed by vigorously pipetting the medium on the plates. FACScan analysis (see below) revealed that over 98% of B cells were depleted by this method. Positively selected populations were >95% B cells. CD4+ or CD8+ cells were also removed by panning the lymphocytes on immunoabsorbent plates onto which monoclonal antibody (MAb) specific for these surface antigens (secreted by the hybridoma cell clone YTS 191.1 or YTS 169.4, respectively; 7) had been absorbed. Since these rat MABs did not bind directly to the immunoabsorbent monolayers, the monolayers were first saturated with rabbit anti rat immunoglobulin G. Then 30 μ l of the rat MABs, as ascitic fluid (Sera-Lab Ltd., Crawley Down, Sussex, United Kingdom) diluted in 5 ml of PBS, was incubated with these plates at 4°C for 12 h with rocking.

In a number of experiments, CD8+ or CD4+ cells were sensitized, for complement lysis and opsonization, with MAb (clone YTS 169.4 or YTS 191.1, respectively) specific for these cell surface antigens (7) before their adoptive transfer. Briefly, 5×10^7 cells were incubated for 15 min at 37°C in 1 ml of antibody (as ascitic fluid; Sera-Lab) that had been diluted 1:50 in PBS that also contained guinea pig complement at a final dilution of 1:40 (Sera-Lab). The cell-antibody mixtures were adoptively transferred to mice (7). In all adoptive transfer experiments, 2×10^7 to 4×10^7 lymphocytes in 200 μ l of PBS were inoculated intraperitoneally into immunodeficient mice.

FACScan analysis. B lymphocytes, CD4+ cells, and CD8+ cells were directly stained with fluorescein isothiocyanate-labeled antibodies specific for mouse immunoglobulin, CD4, and CD8 (Sera-Lab), respectively. The lymphocytes were incubated for 30 to 60 min at 4°C with the antibodies (diluted 1:100) in 100 μ l of PBS and washed once with 10 ml of PBS, and the percentage of fluorescent cells in 10,000 events was determined by using the LYSYS program of a Becton Dickinson FACScan.

Cytotoxicity assays. Spleens were removed from immune mice 4 to 6 weeks after the second immunization by infection. Spleen cells were restimulated in vitro for 3 days with SV5-infected spleen cells as described for the generation of CTLs specific for respiratory syncytial virus (3). Target cells were P815 (*H-2^d*), EL4 (*H-2^b*), or RIE (*H-2^k*) infected with SV5 at a multiplicity of infection of 2 to 5 for 16 to 18 h. Uninfected cells were used as controls for nonspecific lysis. A standard ⁵¹Cr release assay performed in U-bottom microtiter plates was used as described elsewhere (3, 18, 29). Tests were set up in triplicate, using 10^4 target cells per well. The percentage of lysis was calculated as [(sample counts per minute – background counts per minute)/(total counts per minute – background counts per minute)] $\times$ 100, where total counts per minute is the radioactivity released by targets treated with 0.1% sodium dodecyl sulfate (SDS).

Mitogen-induced lymphocyte proliferation. Splenocytes were cultured in 96-well microtiter plates (10^5 cells per well) in 0.2 ml of RPMI 1640 with 2% rat serum, 2 mM glutamine, 10 mM *N*-2-hydroxyethylpiperazine-*N*'-ethanesulfonic acid (HEPES), and 50 μ M β -mercaptoethanol plus various concentrations of either LPS (Sigma) or ConA (Sigma). After 72

h in culture, the cells were radioactively labeled for 16 h by addition of 0.5 μ Ci of [3 H]thymidine to each well. The cells were then harvested on glass fiber paper by using an Ilacon cell harvester, and the amount of radioactive incorporation was estimated by liquid scintillation counting.

Western blot analysis of lung extracts from SV5-infected mice. Lungs were homogenized in SDS-polyacrylamide gel electrophoresis disruption buffer, sonicated by using an MSE ultrasonic probe, and heated for 5 min at 100°C. Particulate material was pelleted by centrifugation (6,000 $\times$ g for 3 min), and the dissociated polypeptides were separated by electrophoresis through a 15% SDS-polyacrylamide slab gel. The separated polypeptides were transferred to nitrocellulose by using a semidry gel electroblotter. The nitrocellulose was then reacted with a pool of MABs to the P protein (19), and bound antibody was detected by 125 I-labeled protein A and autoradiography as previously described (20).

Titration of virus in infected mouse lungs. At various times postinfection, the mice were sacrificed and the lungs were removed. Lungs were homogenized in growth medium by using an MSE overhead homogenizer and sonicated with an ultrasonic probe, and the particulate material was removed by centrifugation (6,000 $\times$ g for 5 min). The amount of infectious virus was titrated by infecting Vero cells, grown in 96-well microtiter plates, with 100 μ l of doubling dilutions of the lung suspension in growth medium.

Preparation of radiolabeled antigen extracts, immunoprecipitation, and SDS polyacrylamide gel electrophoresis. The methods used have been described elsewhere (19). In the preparation of soluble antigen extracts, the immunoprecipitation buffer used consisted of 10 mM Tris hydrochloride (pH 7.2), 5 mM EDTA, 0.5% Nonidet P-40, 0.1% SDS, 0.65 M NaCl, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 10 mM Na₂S₂O₅).

Neutralization test. Twofold dilutions of sera (100 μ l), diluted in tissue culture medium containing 2% calf serum, were incubated at 37°C for 2 h with 100 μ l of SV5 (5 $\times$ 10⁵ PFU/ml). The antibody-virus mixtures were then used to infect Vero cells growing as monolayers in 96-well microtiter plates. The cells were incubated at 37°C for an additional 30 to 40 h. The cells were then fixed with 5% formaldehyde–2% sucrose in PBS for 10 min, permeabilized with 0.5% Nonidet P-40–10% sucrose in PBS for 5 min, and washed three times with PBS. Virus antigens were detected by incubating the cells with a mixture of MABs specific for SV5 (as 1:500 dilutions of ascitic fluids in PBS), and bound antibody was detected with 125 I-labeled protein A as described by Randall et al. (19).

In situ hybridization. Frozen mouse lungs were sectioned by using a Bright OTF/AS cryostat. Sections (10 μ m thick) were fixed in ethanol-acetic acid (3:1, vol/vol) for 15 min at room temperature, washed with absolute ethanol for 5 min, and stored desiccated at –20°C until required. Before hybridization, the sections were pretreated with 0.2 M HCl and proteinase K to increase diffusion of the probe (11). 35 S-labeled single-stranded DNA probes specific for the HN gene of SV5 were prepared by primer extension and excision of the HN gene (obtained originally from R. Lamb, Northwestern University, Evanston, Ill., as an insert in pBR322; 16) cloned into M13, followed by purification of the single-stranded probe on a 2% low-melting-point agarose gel (12). Hybridization conditions used for binding the single-stranded DNA probe to lung sections were based on those described by Haase et al. (11) for hybridization to RNA.

FIG. 1. Autoradiogram of a Western blot used to detect the presence of the P protein of SV5 in lung extracts of immunodeficient mice infected with SV5 for 6 and 10 days (tracks 1 to 4). Also shown is the amount of antigen present in groups of mice to which 4×10^7 nonimmune (tracks 5 to 8) or immune (tracks 9 to 12) lymphocytes were adoptively transferred 2 h after infection with SV5.

Bound probes were detected by autoradiography, using Ilford K5 photographic emulsion.

RESULTS

SV5 infection of immunodeficient mice. SV5 inoculation of mice made immunodeficient by X irradiation resulted in a prolonged infection. Similar amounts of virus proteins were detected by Western blot analysis in the lungs at 6 and 10 days (Fig. 1, tracks 1 to 4) postinfection, and virus was detected in the lungs at 19 days postinfection both by Western blot analysis (data not shown) and by in situ hybridization (Fig. 2). However, although there was a substantial increase in the amount of virus proteins and nucleic acid present in the lungs, the levels of infectious virus remained relatively low and never reached the level of input virus (Table 1). Nevertheless infectious virus was recovered from the lungs at 19 days postinfection. Although immunodeficient mice failed to clear the virus rapidly, they did not show signs of overt disease. Furthermore, in situ hybridization (Fig. 2) and immunofluorescence (data not shown) analyses demonstrated that there was not extensive spread of virus within infected lungs. The infection was eventually cleared in immunodeficient mice (by 28 days postinfection), probably because they eventually mount an immune response to SV5. Thus, these results suggest that SV5 establishes a persistent infection in mice in the absence of an effective immune response to the virus.

Clearance of virus from lungs of immunodeficient mice. In immunocompetent mice, no viral proteins (20) or infectious virus (Table 1) was detected after 7 days postinfection. Indeed, after infection of immunocompetent mice, both B and T memory cells were generated; these cells were detected either indirectly by immune transfer experiments (see below) or, in the case of CTLs, by standard chromium release assays in vitro. Thus, splenocytes isolated from immunocompetent mice 4 weeks after SV5 infection were capable of killing virus-infected cells in an MHC-restricted manner. For example, immune splenocytes isolated from BALB/c (*H-2^d*) mice after restimulation in culture for 3 days killed SV5-infected P815 (*H-2^d*) cells but not uninfected P815 cells. They also failed to kill infected or uninfected target cells that had an MHC haplotype different from that of BALB/c cells (Fig. 3). Since P815 cells express class I but not class II MHC antigens, the killing observed was mediated through class I-restricted CTLs.

To further examine the role of immune lymphocytes in

FIG. 2. Detection of SV5 in lungs of immunodeficient mice by in situ hybridization. Mice were infected intranasally with SV5 and sacrificed at 5, 10, 14, and 19 days postinfection. Lungs were removed, frozen in liquid nitrogen, and sectioned with a cryostat. The presence of SV5 RNA in the sections was detected with a single-stranded DNA probe specific for the HN gene.

clearing SV5 from lungs, a series of experiments was carried out in which splenocytes isolated from immune or nonimmune mice were transferred to immunodeficient mice infected with SV5. These experiments demonstrated that immune lymphocytes but not nonimmune lymphocytes cleared the virus infection by 10 days postinfection (Fig. 1). In these initial experiments, unselected immune cells, including B and T lymphocytes, were transferred to the immunodeficient mice. At 10 days posttransfer the spleens of

these animals were atrophied but the small amounts of lymphocytes isolated ($\sim 4 \times 10^6$ to 6×10^6 cells per spleen) showed strong activation by the T-cell mitogen ConA and weak activation by the B-cell mitogen LPS (data not shown). Nevertheless, analysis of the antibody response revealed that mice which had received immune lymphocytes were synthesizing large amounts of neutralizing antibody by 10 days postinfection, but mice that had received nonimmune lymphocytes were not (Table 2). Even by 6 days postinfection, a small amount of neutralizing antibody could be detected in a number of mice after transfer of immune lymphocytes. It should be noted that one of the mice which received immune lymphocytes had not cleared the infection by 10 days postinfection (Fig. 1, mouse 9). However, since this mouse did not make an antibody response to SV5 (Table 2, mouse 9), this result suggested that the adoptive transfer had been unsuccessful. Consequently, when possible we used measurements of the antibody response as a rough guide to how successful the adoptive transfers had been.

Our previous studies in immunocompetent mice had suggested that there was no correlation between the level of serum-neutralizing antibody and the speed of clearance of the virus from the lungs (20). To answer the question of whether serum-neutralizing antibody plays a major role in clearing SV5 infections from mouse lungs, two series of experiments were carried out. We had previously isolated a large bank of MABs to the HN protein of SV5. All of the antibodies were capable of neutralizing virus infectivity and defined four different antigenic sites on the HN protein. We had also isolated a MAB to the F protein of SV5 that

TABLE 1. Recovery of SV5 from lungs of immunocompetent and immunodeficient mice^a

Mouse no.	Log ₁₀ titer/lung at given days postinfection ^a								
	1	2	3	4	6	7	10	14	19
Immunocompetent									
1	4.0	3.4	3.1	3.4	3.1	1.4			
2	4.0	3.7	3.1	3.4	3.1	<1			
3	4.3	4.0	3.4	3.1	3.4	1.4			
Immunodeficient									
1	3.9				3.3		3.9	3.0	3.6
2	4.8				2.4		3.6	2.7	3.3
3	4.3				2.7		2.7	2.7	3.3
4	ND				2.7		ND	4.2	2.7

^a Groups of immunocompetent and immunodeficient mice were infected intranasally with SV5. At various times postinfection, mice were sacrificed and the titers of virus in their lungs were estimated.

^b The values for all mice 10 min after infection were >5 ; values for immunocompetent mice on days 5 and 8 were 3.4 and <1 , respectively. ND, Not determined.

FIG. 3. Detection of CTLs in splenocytes isolated from groups of six immune BALB/c ($H2^{-d}$) mice (a) or nonimmune mice (b). CTL cultures and chromium release assays were set up as described in Materials and Methods. Target cells were P815 cells ($H2^{-d}$) infected with SV5 (□), EL4 cells ($H2^{-b}$) infected with SV5 (△), uninfected P815 cells (■), and uninfected EL4 cells (▲).

neutralized virus infectivity (19). A pool of these antibodies was made and passively transferred both before and after infection of immunodeficient mice. This experiment was repeated multiple times. Although there were slightly reduced amounts of virus antigen in the mice that received the pool of MAbs, these experiments clearly demonstrated that the presence of high-titer serum antibody did not have a major influence on the rate of clearance of SV5 from infected lungs (Fig. 4) despite the facts that (i) both anti-HN and anti-F antibodies were detected by immunoprecipitation at the time of sacrifice of these animals (Fig. 5) and (ii) the measurable levels of serum-neutralizing antibody were higher, both immediately after passive transfer and at the time of sacrifice of the animals, than those in the mice which successfully cleared the virus infection after transfer of immune lymphocytes (compare Tables 2 and 3).

The inability of neutralizing antibody alone to play a major role in clearing virus was confirmed by purifying B cells from immune spleens and transferring them to immunodeficient mice immediately after infection with SV5. The B cells were purified by panning immune lymphocytes on immunoabsorbent monolayers to which sheep anti-mouse immunoglobulin

had been bound (17). This purification procedure resulted in a population of lymphocytes that were more than 97% pure for B cells as determined by FACScan analysis. A total of 4×10^7 purified B lymphocytes were transferred intraperitoneally to immunodeficient mice. After infection with SV5, these mice made very high levels of neutralizing antibody (1/1,280 to 1/5,120). However, they failed to clear the virus infection (Fig. 6).

From these and our previous results, it appeared likely that T cells were responsible for the clearance of virus. This supposition was supported by the finding that immune spleen cells that had been depleted of adherent cells and 95% of B cells by panning were still capable of clearing SV5 from the lungs of immunodeficient mice (data not shown; Fig. 7). To further examine the role of T lymphocytes in clearing SV5 infections, two series of adoptive transfer experiments were carried out. In the first series of experiments, CD4+ or CD8+ cells were sensitized for complement lysis and opsonization upon adoptive transfer by treating the lymphocytes with MAbs specific for these antigens before the transfer. This method has been shown to prevent graft-versus-host reactions (6), and it is known that transfer of the antibodies alone depletes the respective populations of cells in vivo (7). Although some degree of protection was still observed after treatment of the cells with the anti-CD8 MAb, it was significantly reduced. However, treatment of the lymphocytes with the MAb to CD4 had no measurable effect

TABLE 2. Neutralization titers to SV5 in sera of immunodeficient mice, tested as described for Fig. 1, at the time of sacrifice

Mouse no. ^a	Neutralization titer ^b
Day 6 postinfection	
9.....	<20
10.....	<20
11.....	40
12.....	40
Day 10 postinfection	
9.....	<20
10.....	320
11.....	320
12.....	640

^a Corresponding to the lanes in Fig. 1.
^b Values for mice 1 to 8 on both days were <20.

FIG. 4. Autoradiogram of a Western blot used to detect the presence of the P protein of SV5 in lung extracts of immunodeficient mice at 10 days postinfection. Lung extracts were prepared from control mice (tracks 1 to 5) or from mice that had received a pool of MAbs to the HN and F proteins either on the day before infection (tracks 6 to 9) or 2 h postinfection (tracks 10 to 14).

FIG. 5. Analysis of [35 S]methionine-labeled polypeptides, separated by electrophoresis through a 12% SDS-polyacrylamide gel, from the immunoprecipitates formed by the reaction of soluble antigen extracts of SV5-infected BHK cells with a pool of MAbs to the HN, F, NP, P/V, and M proteins (track 1) or MAb to the F (track 2) or HN (track 3) protein. Track 4 demonstrates the presence of antibodies specific for the HN and F proteins in pooled sera taken at the time of sacrifice from immunodeficient mice (Fig. 4, tracks 6 to 9) to which MAbs to the HN and F proteins had been passively transferred before infection with SV5. The sera of control mice (Fig. 4, tracks 1 to 5) had no detectable antibody to SV5 (data not shown).

(Fig. 8). In these experiments the B cells were not removed, and it is pertinent to note that the mice which received the lymphocytes that had been treated with the anti-CD8 MAb had the highest levels of neutralizing antibody (Table 4) but were the least protected.

In the second series of experiments, different lymphocyte populations were removed from immune spleen cells by panning on immunoaffinity plates. Initially more than 95% of B lymphocytes were removed by this method, and subsequently either CD4⁺ or CD8⁺ cells were depleted. An analysis of the resulting lymphocyte populations is shown in Fig. 9. Immune cells from which more than 95% of B cells and CD4⁺ cells had been removed still efficiently cleared the virus infection. However, immune cells that were depleted of more than 95% B cells and CD8⁺ cells also enhanced the speed of clearance of virus, albeit to a lesser degree (Fig. 7). From these results, it was not clear whether the small degree

TABLE 3. Neutralization titers to SV5 in sera of immunodeficient mice, tested as described for Fig. 4, at the time of sacrifice

Mouse no. ^a	Neutralization titer ^b
6	1,280
7	1,280
8	320
9	1,280
10	>2,560
11	2,560
12	2,560
13	2,560
14	1,280

^a Corresponding to the lanes in Fig. 4.

^b Values for mice 1 to 5 were >20.

FIG. 6. Autoradiogram of a Western blot used to detect the P protein of SV5 in lung extracts of immunodeficient mice at 10 days postinfection. Lung extracts were prepared from control mice (tracks 1 to 3) or from mice to which unselected immune lymphocytes (tracks 4 to 6) or purified immune B lymphocytes (tracks 8 to 11) were adoptively transferred 2 h after infection.

of protection observed was due to CD4⁺ effector cells or low levels of contamination with CD8⁺ cells. Nevertheless, both sets of adoptive transfer experiment showed that CD8⁺ cells were the predominant cell type involved in clearing SV5 from infected mouse lungs.

DISCUSSION

In this report, we show that SV5 establishes a prolonged infection in immunodeficient mice. Although there was a significant increase in both viral RNA and proteins, little infectious virus was produced. A similar situation occurs after infection of mouse cells *in vitro* in that there is an increase in the levels of both viral RNA and protein yet no infectious virus is produced. Furthermore, the virus establishes a persistent infection in these cells, since the virus can be detected for prolonged periods (>21 days) yet the cells remain viable (F. Heaney and R. E. Randall, unpublished observations). Thus, the prolonged infection *in vivo* can best be explained if similarly SV5 establishes a nonlethal persistent infection rather than an acute infection in mouse lung cells. In the presence of an active immune response, either such cells would then be eliminated or virus replication would be inhibited by the release of antiviral lymphokines.

Adoptive transfer of immune lymphocytes to immunodeficient mice enhanced the speed with which these animals cleared the SV5 infection. Depletion of CD8⁺ cells but not B cells from immune splenocytes dramatically reduced their ability to clear the virus infection. These results demonstrated that virus clearance was primarily mediated by CD8⁺ effector cells. However, in these studies it appeared that CD4⁺ cells also had some antiviral activity. *In vivo*, the T cells could mediate their activity either directly through their cytolytic activities or through the production of antiviral lymphokines. The demonstration of class I-restricted

FIG. 7. Autoradiogram of Western blot used to detect the P protein of SV5 in lung extracts of immunodeficient mice at 10 days postinfection. Lung extracts were made either from control mice (tracks 1 to 3) or from mice to which unselected immune lymphocytes were adoptively transferred 2 h after infection with SV5 (tracks 4 to 6). The ability of immune lymphocytes that had been depleted of B cells and CD4⁺ (tracks 7 to 9) or CD8⁺ (tracks 10 to 12) lymphocytes (see also Fig. 9) to clear SV5 from infected lungs of immunodeficient mice was also examined.

FIG. 8. Autoradiogram of a Western blot used to detect the P protein of SV5 in lung extracts of immunodeficient mice at 10 days postinfection. Lung extracts were made either from control mice (tracks 1 to 3) or from mice to which unselected immune lymphocytes were adoptively transferred 2 h after infection with SV5 (tracks 4 to 7). The ability of immune lymphocytes that had been sensitized with MAb to CD4 (tracks 8 to 11) or CD8 (tracks 12 to 15) to clear SV5 from infected lungs of immunodeficient mice was also examined.

CTL activity *in vitro* suggests that *in vivo* CD8⁺ cells may also mediate their antiviral effect directly through their cytolytic activity. With regard to the role of antibody in this system, the presence of serum-neutralizing antibody in immunodeficient mice had little effect on the speed of clearance observed. The small effect observed may be explained if neutralizing antibody inactivated any infectious virus produced during the infection, thus reducing the spread of virus in the lungs, rather than being involved in the elimination of persistently infected cells. The observation that serum-neutralizing antibody had little effect in the clearance of SV5 from infected mouse lungs is at odds with multiple studies demonstrating that transfer of neutralizing MAbs can protect experimental animals from infection with other paramyxoviruses, as measured by infectious titer assays (8, 13, 23, 28). Part of the explanation may be that in these latter systems the infection is productive, with large amounts of infectious virus being produced. Obviously, in such systems neutralizing antibody may well prevent spread of virus within infected tissues and hence be protective.

TABLE 4. Neutralization titers to SV5 in sera of immunodeficient mice, tested as described for Fig. 8, at the time of sacrifice

Mouse no. ^a	Neutralization titer ^b
4	1,280
5	320
6	160
7	160
8	80
9	320
10	160
11	160
12	1,280
13	320
14	640
15	640

^a Corresponding to the lanes in Fig. 8.

^b Values for mice 1 to 3 were >10.

However, since we have previously noted (20) that high levels of serum-neutralizing antibody can inactivate virus *in situ* upon extraction of lung material, it would be of interest in these studies to measure the level of virus load in infected lungs by methods other than infectious titer assays.

These results are similar to those reported by Cannon et al. (5) on the clearance of a persistent respiratory syncytial virus infection from infected mice. These authors also concluded that CD8⁺ (Lyt2⁺) cells were more effective than CD4⁺ (L3T4⁺) cells in clearing the infection. Furthermore, since no respiratory syncytial virus-specific antibody was detected at a time when the infection was cleared, these authors suggested that clearance was by an antibody-independent mechanism. However, they did make an additional

LYMPHOCYTES STAINED WITH

FIG. 9. FACSscan analysis of the proportion of B cells, CD4⁺ cells, and CD8⁺ cells in an unselected population of immune spleen cells and in populations of spleen cells that had been depleted of B cells and either CD4⁺ or CD8⁺ lymphocytes by panning on immunoaffinity monolayers. The ability of these different lymphocyte populations to clear SV5 infection from lungs of immunodeficient mice was examined, and the results are shown in Fig. 7.

observation, which seems difficult to explain, that after delayed transfer (14 days postinfection) of primed lymphocytes to infected animals, protection appeared to correlate with antibody production rather than level of CTLs.

To establish a persistent infection *in vivo*, a virus must avoid elimination by the host immune response. The results presented here have shown that persistent paramyxovirus infections can be maintained in the presence of high-titer neutralizing antibodies. It is thus clear that T cells play a critical role in clearing both acute and persistent paramyxovirus infections. Given that T cells may potentially recognize target sites on any virus protein (reviewed in reference 17a), there has to be some explanation for how these viruses establish persistent infections in the presence of an immune response capable of controlling an initial acute infection. One possible explanation is that persistent infections occur at immunologically privileged sites. However, this is unlikely to be the complete explanation. Since MHC antigens are extremely polymorphic (22), different individuals in a heterologous population may recognize different T-cell target antigens, depending on their histocompatibility status (2, 4, 10, 15). Consequently, it may be that persistent infections develop in individuals with specific human leukocyte antigen repertoires. As shown here and elsewhere (Randall and Russell, *in press*), in persistent paramyxovirus infections the virus genome must be transcribed and replicated, even though little or no infectious virus need be produced. Therefore, the virus must retain the capacity to encode those virus proteins (NP, P/V, and L) involved in transcription or replication. However, mutations or deletions in other virus-specified proteins (HN, F, and M) may be tolerated. Thus, it may be that persistent paramyxovirus infections do not become established in individuals whose effector T lymphocytes recognize target antigens on virus proteins critical for the maintenance of the persistent infection. On the other hand, individuals who can produce only effector T-cell responses against target antigens on other virus proteins, e.g., the surface glycoproteins or matrix protein, may not be able to clear a persistent virus infection if mutations or deletions arise in these virus genes.

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