Solid Matrix-Antibody-Antigen Complexes Induce Antigen-Specific CD8⁺ Cells That Clear a Persistent Paramyxovirus Infection

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Received 6 August 1990/Accepted 5 November 1990

We have previously shown that the adoptive transfer of splenocytes, isolated from mice immunized by infection with the paramyxovirus simian virus 5 (SV5), enhance the speed of clearance of SV5 from the lungs of immunodeficient mice; clearance is mediated primarily through CD8⁺ effector cells and not by serum neutralizing antibody (D. F. Young, R. E. Randall, J. A. Hoyle, and B. E. Souberbielle, *J. Virol.* 64:5403-5411, 1990). In this article we demonstrate that immunization of mice with solid matrix-antibody-antigen (SMAA) complexes also induces CD8⁺ effector cells that are responsible for clearing persistent SV5 infections in immunodeficient mice. The demonstration that immunization with SMAA complexes (an exogenous antigen) can induce class I-restricted cytotoxic T lymphocytes (CTLs) suggests that these cells may be responsible for virus clearance *in vivo*. This premise is supported indirectly by the observation that immunization with SMAA complexes was less efficient in inducing class I-restricted CTLs (as measured *in vitro*) than was infectious virus and that splenocytes isolated from mice immunized with SMAA complexes were also less efficient in clearing virus from lungs of immunodeficient mice than were splenocytes isolated from mice immunized by infection with virus. This was not because the SMAA complexes were generally less immunogenic than infectious virus, since mice immunized with SMAA complexes (which contained the HN protein of SV5) produced higher levels of neutralizing antibody than mice immunized with infectious virus. In the majority of experiments, fixed and killed suspensions of *Staphylococcus aureus* Cowan strain A were used as the solid matrix in the construction of SMAA complexes. However, in this article we present evidence that alum-antibody-antigen complexes are as immunogenic as *S. aureus* A-antibody-antigen complexes. These results suggest that the immunological reactivity of the solid matrix itself does not influence the intensity of the immune response to the antigens of interest in the SMAA complexes. The significance of these results for vaccine design are discussed.

Antibodies and T lymphocytes play critical roles in prevention of and recovery from viral infections. Effector antibody responses are primarily concerned with the inactivation, or neutralization, of free virus. Cytotoxic T cells (CTLs) recognize and kill infected cells, thus reducing or preventing the production of progeny virus. In addition, T cells may release antiviral lymphokines, such as gamma interferon, that inhibit virus replication. While neutralizing antibodies interact with antigens located on the surface of the virus particle, T cells may potentially recognize target antigens on any virus protein (for a review of how antigens are recognized by B and T lymphocytes, see reference 25). In order to induce long-lived protective immunity by vaccination against certain virus diseases, it may therefore be necessary to induce a broad immune response with the expansion of both B- and T-cell clones that recognize many different target antigens on different virus proteins. Consequently, in such situations it may be necessary to incorporate multiple antigens into a vaccine. We have suggested that one way of constructing such vaccines may be through the design and manufacture of solid matrix-antibody-antigen (SMAA) complexes (24, 26, 27).

T lymphocytes recognize virus antigens, as peptides, in association with either class I or class II major histocompatibility (MHC) antigens. In general, CTLs recognize virus antigens in association with class I MHC molecules, while T helper/inducer cells recognize antigens in association with class II MHC molecules (for review, see reference 3). Thus,

in the majority of virus infections, class I-restricted CTL responses are the predominant response (6-8, 12, 21, 39), although for some viruses it appears that class II-restricted CTLs may also be important (16, 17, 20, 30, 36). 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 (31).

It is well documented that exogenous antigen is normally processed through an endosomal pathway for class II MHC presentation (1). However, there is a body of evidence which suggests that exogenous antigens (e.g., noninfectious virus antigens) are not normally processed in a way which leads to the successful induction of class I-restricted CTLs, whereas endogenous antigens (e.g., virus proteins made in infected cells) are (9, 13, 23). This may be because for virus antigens to become associated with class I MHC molecules, they have to be processed within the cytosol of antigen-presenting cells (22, 34, 37). Nevertheless, it has been reported that class I-restricted CTLs can be generated *in vivo* after immunization with noninfectious virus antigens, e.g., recombinant hepatitis B virus vaccine (18), the NP protein of influenza virus (35), and a hybrid influenza virus HA protein (19). Class I-restricted CTL induction has also been described with ovalbumin as the immunizing antigen (32). More recently, Takahashi et al. (33) have reported the induction of CD8⁺ cytotoxic T cells by immunization with immunostimulating complexes (ISCOMS) containing the gp160 protein of human immunodeficiency virus. Our own data on immunization with SMAA complexes containing

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simian virus 5 (SV5) antigens demonstrated that lymphocytes isolated from mice immunized with such complexes were capable of killing SV5-infected P815 cells, a cell line that does not express class II MHC molecules, suggesting that immunization with SMAA complexes can induce class I-restricted CTLs (26).

We have previously demonstrated that immunization of mice with SMAA complexes containing either internal or surface structural proteins of the paramyxovirus SV5 enhances the speed of clearance of the virus from the lungs of infected immunocompetent mice (29). There was no correlation between the speed of clearance and the level of serum neutralizing antibody. We have extended these observations, demonstrating that indeed serum neutralizing antibodies play only a minor role in clearing the virus infection. Furthermore, by the adoptive transfer of immune splenocytes isolated from mice previously infected with SV5 to immunodeficient mice, we demonstrated that in this system virus clearance was primarily dependent on CD8⁺ effector cells (38). Here, we demonstrate that immunization with SMAA complexes can induce class I-restricted CTLs and that the clearance observed following transfer of immune lymphocytes isolated from mice immunized by SMAA complexes is also mediated by CD8⁺ effector cells.

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's modification of Eagle's tissue culture medium containing 10% newborn calf serum (growth medium). P815 and EL4 cells were cultivated in 75-cm² tissue culture flasks in growth medium. A human isolate of SV5 (LN [14]) was grown and titrated under appropriate conditions in Vero or BHK cells in medium containing 2% newborn calf serum.

X-irradiation of mice. BALB/c mice (6 to 8 weeks old) were exposed to 5 Gy of whole-body X-irradiation prior to infection with SV5. Immediately following X-irradiation of mice, their splenocytes failed to respond to the B-cell mitogen lipopolysaccharide and the T-cell mitogen concanavalin A. Ten 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, versus 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 concanavalin A but none by lipopolysaccharide. X-irradiated mice do however begin to recover their ability to mount an immune response 2 to 3 weeks after irradiation (2), and by 10 weeks after irradiation the spleens had increased in size so that 2×10^7 to 4×10^7 cells could be isolated per spleen and the splenocytes proliferated in the presence of both lipopolysaccharide and concanavalin A (unpublished observations).

Infection of mice. Mice, while anesthetized with ether, 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 killed and the lungs were removed, weighed, and frozen at -70°C until required.

Production of SMAA complexes and immunization of mice. The production of SMAA complexes with fixed and killed suspensions of *Staphylococcus aureus* Cowan strain A as the solid matrix has been reported in detail elsewhere (26, 29). Here we describe how alum-antibody-antigen (alum-Ab-Ag) complexes were prepared. Alum precipitates were made

by mixing 2 volumes of 10% potassium aluminum sulfate solution with 1 volume of 1 M sodium bicarbonate while vortexing, and the precipitate was pelleted by centrifugation at $500 \times g$ for 5 min [500 μ l of NaHCO₃ plus 1,000 μ l of Al₂(SO₄)₃ · K₂SO₄ · 24H₂O gives approximately 160 mg of a wet alum precipitate]. Alum-Ab complexes were made by incubating the alum with saturating amounts of purified monoclonal antibodies (MAbs) in phosphate-buffered saline (PBS) overnight at 4°C (20 mg of wet alum precipitate is saturated by $\sim 25 \mu$ g of purified antibody). Alum-Ab-Ag complexes were prepared as described previously for preparing *S. aureus* strain A-antibody-antigen (St. A-Ab-Ag) complexes (26, 29). Briefly, a soluble antigen extract of SV5-infected BHK cells was made as described previously (28). These extracts were then mixed continuously with the alum complexes (15 mg of a wet alum complex was mixed with $\sim 2 \times 10^8$ cell equivalents of antigen extract) for 4 h at 4°C . The resulting complexes were washed three times by sedimentation ($3,300 \times g$ for 3 to 10 min) from and resuspension in immunoprecipitation buffer (20 mM Tris hydrochloride [pH 7.2], 5 mM EDTA, 0.5% Nonidet P-40, 0.1% sodium dodecyl sulfate [SDS], 0.65 M NaCl) and then twice with PBS. It should be noted that antibody bound to alum (unlike when bound to *S. aureus*) slowly leaches off the alum in immunoprecipitation buffer. However, the binding of antigen to antibody stabilizes the complex. Consequently, when making alum-Ab-Ag complexes, it is very important to have high levels of antigen in the soluble antigen extract so that the antigen is bound to the antibody before the antibody leaches off the alum (32a). The purity and quantity of antigen bound to St. A-Ab-Ag or alum-Ab-Ag complexes were estimated by SDS-polyacrylamide gel electrophoresis (PAGE) on minislab gels (26, 29) (see Fig. 1 and 5). Mice were immunized twice intraperitoneally (i.p.) with 2 to 5 μ g of antigen presented as alum-Ab-Ag complexes or St. A-Ab-Ag complexes. When mice were immunized with more than one virus protein, e.g., HN, NP, P, and M, the proteins were purified as separate SMAA complexes, which were then mixed together prior to immunization. A gap of 3 to 4 weeks was left between the first and second immunizations, and 10 to 14 days after the second immunization the mice were bled, and the sera were collected and stored at -20°C until required.

Adoptive transfer of different lymphocyte populations to immunodeficient mice. BALB/c (*H-2^d*) mice were immunized with either SMAA complexes (i.p.) or infectious virus (by the nasal route) as described above. Spleen cells were isolated from immune mice 4 to 6 weeks after the second immunization by standard methods. From 2×10^7 to 4×10^7 unselected or selected (below) lymphocytes were adoptively transferred by i.p. inoculation in PBS. CD8⁺ or CD4⁺ cells were sensitized for complement lysis and opsonization with MAbs (clones YTS 169.4 and YTS 191.1, respectively) specific for these cell surface antigens (10, 11) prior to 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, Crawley Down, England) that had been diluted 1:50 in PBS which also contained guinea pig complement at a final dilution of 1:40 (Sera-Lab). The cell-antibody mixtures were adoptively transferred to mice (10, 11). In the adoptive transfer experiments, 1×10^7 to 4×10^7 lymphocytes (see figure legends) in 200 μ l of PBS were inoculated i.p. into immunodeficient mice.

FACS analysis. B lymphocytes and CD4⁺ and CD8⁺ cells were directly stained with fluorescein isothiocyanate-labeled antibodies specific for mouse immunoglobulin, CD4,

or CD8 (Sera-Lab). The lymphocytes were incubated with the antibodies (diluted 1:100) in 100 μ l of PBS for 30 to 60 min at 4°C 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 on a Becton Dickinson FACScan.

Cytotoxicity assays. Spleens were removed from immune mice 4 to 6 weeks after their second immunization. Splenocytes were restimulated in vitro twice (at the time the cultures were set up and after 5 days in culture) with SV5-infected spleen cells in the manner described for the generation of CTLs specific for respiratory syncytial virus (5). The level of CTL activity was measured in these cultures in 4- or 10-day cultures. Target cells were P815 (*H-2^d*) or EL4 (*H-2^b*) cells infected with SV5 at 2 to 5 PFU/cell for 16 to 18 h. Uninfected cells were used as controls for nonspecific lysis. A standard ^{51}Cr release assay, performed in U-bottomed microtiter plates, was based on methods described in detail elsewhere (5, 26). Tests were set up in triplicate with 10^4 target cells per well. The percent lysis was calculated as [(sample release - background release)/(total release - background release)] $\times$ 100, where total release is the radioactivity released by targets treated with 0.1% SDS.

Preparation of radiolabeled antigen extracts, immunoprecipitation, and SDS-PAGE. The methods for these techniques have been described elsewhere (28). In the preparation of soluble antigen extracts, the immunoprecipitation buffer used consisted of 20 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 $\text{Na}_2\text{S}_2\text{O}_5$). The polypeptides present in SMAA complexes were analyzed by electrophoresis through thin (0.4 mm) minislab gels (4 by 8 cm) with a Uniscience (London, United Kingdom) mini vertical electrophoresis system. Polypeptides separated for Western immunoblot analysis were separated through larger polyacrylamide slab gels (14 by 18 cm) of greater thickness (2 mm).

Western blot analysis of lung extracts from SV5-infected mice. Lungs were homogenized in SDS-PAGE disruption buffer (28), sonicated with 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 with a semidry gel electroblotter (LKB). The nitrocellulose was then reacted with a pool of MAbs to the P protein (28), and bound antibody was detected by ^{125}I -labeled protein A and autoradiography as described previously (29).

Neutralization test. Twofold dilutions of sera (100 μ l) in tissue culture medium containing 2% newborn calf serum were incubated at 37°C for 2 h with 100 μ l of SV5 (5×10^5 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 a further 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. (28).

FIG. 1. SDS-PAGE analysis (Coomassie brilliant blue-stained gel) of the purification of the HN (track 1), NP (track 2), P (track 3), and M (track 4) proteins of SV5 from soluble antigen extracts of infected cells by St. A-Ab complexes. Polypeptides were separated by electrophoresis through a 12% SDS-polyacrylamide minislab gel. The positions of the HN, NP, M, and P proteins are shown on the left side of the figure, and the positions of the antibody heavy (IgH) and light (IgL) chains are shown on the right.

RESULTS

Induction of class I-restricted T cells with SMAA complexes.

Mice were immunized either by prior infection with SV5 or by injection with mixtures of St. A-Ab-Ag complexes (exogenous antigen) containing the HN, NP, P, and M proteins of SV5 (Fig. 1). Splenocytes isolated from mice immunized either by infection with SV5 or with SMAA complexes were capable of killing virally infected cells in an MHC-restricted manner as assayed in standard chromium release tests. Thus, immune splenocytes isolated from BALB/c mice (*H-2^d*) following restimulation in culture killed SV5-infected P815 (*H-2^d*) cells but not uninfected P815 cells. They also failed to kill infected or uninfected target cells which had a different MHC haplotype than BALB/c cells (Fig. 2). Since P815 cells express class I MHC antigens but not class II MHC antigens, these results confirm that immunization with SMAA complexes can induce class I-restricted CTLs.

Interestingly, however, the level of CTL activity in animals immunized by prior infection with SV5 was greater than that in mice immunized with SMAA complexes. Indeed, while high levels of CTL activity could be detected in 4-day-stimulated cultures of splenocytes isolated from mice immunized by infection with virus, only low levels of CTL activity could be detected in splenocytes isolated from animals immunized with SMAA complexes. However, in agreement with our previous results (29), high levels of CTL activity were detected in 10-day-restimulated cultures of splenocytes isolated from mice immunized with SMAA complexes (Fig. 2).

Clearance of SV5 from the lungs of immunodeficient mice by adoptive transfer of immune lymphocytes. Following infection of mice made immunodeficient by X-irradiation, SV5 establishes a persistent infection in which virus can be detected in the lungs at 20 days postinfection. Adoptive transfer of splenocytes isolated from mice previously infected with SV5 enhances the speed of clearance of virus from such mice, and the rate of clearance is dependent on the presence of CD8⁺ cells and not neutralizing antibody (38). To determine whether lymphocytes isolated from mice immunized with SMAA complexes could also enhance the speed of clearance of virus from immunodeficient mice, a series of adoptive transfer experiments were carried out. In the first of these experiments, splenocytes were isolated from mice immunized with the St. A-Ab complexes alone or St. A-Ab-Ag complexes containing the NP protein of SV5.

FIG. 2. Detection of CTLs in spleens taken from groups of four BALB/c (*H-2^d*) mice immunized either with infectious virus (a), with St. A-Ab complexes alone (c and e), or with St. A-Ab-Ag complexes containing the HN, NP, P, and M proteins of SV5 (b and d). Splenocyte bulk cultures were restimulated in vitro on days 1 and 5, and the CTL activity was measured in 4-day (a, b, and c) and 10-day (d and e) cultures. CTL cultures and chromium release assays were set up as described in Materials and Methods. Target cells were P815 cells (*H-2^d*) infected with SV5 (□), EL4 cells (*H-2^b*) infected with SV5 (△), uninfected P815 cells (■), and uninfected EL4 cells (▲).

These cells were transferred to immunodeficient mice immediately after they had been infected with virus. Animals were sacrificed 10 days postinfection, and the level of virus in their lungs was estimated by Western blot analysis with a pool of MAbs specific for the P protein. Only splenocytes isolated from mice immunized with St. A-Ab-NP complexes enhanced the speed of virus clearance. Furthermore, virus clearance occurred in the absence of detectable levels of serum neutralizing antibody (data not shown).

In order to identify the lymphocytes responsible for virus clearance, CD4⁺ and CD8⁺ cells were specifically depleted from immune splenocytes by complement lysis. In these experiments, although untreated splenocytes isolated from mice immunized with St. A-Ab-Ag complexes (containing the HN, NP, P, and M proteins of SV5; Fig. 1) enhanced the speed of clearance of virus from the lungs of immunodeficient mice, depletion of CD8⁺ cells abolished the protection observed (Fig. 3). It is also of note that mice which received immune splenocytes depleted of CD8⁺ cells made higher levels of serum neutralizing antibody than mice which received untreated immune splenocytes that enhanced the speed of clearance of virus (data not shown).

The majority of B-cell and CTL responses are T helper-cell dependent. Consequently, to achieve clonal expansion of CTLs following antigen stimulation, as well as interacting with their appropriate antigen, CTLs usually have to be activated with soluble factors (lymphokines) secreted by antigen-activated T helper lymphocytes (4, 15). Following adoptive transfer of splenocytes isolated from mice immunized with SMAA complexes, it was therefore possible that the presence of CD4⁺ helper cells stimulated nonimmune,

naive CD8⁺ effector cells in vivo, which were responsible for the clearance observed.

To examine this possibility, a series of lymphocyte mixing experiments were performed. CD8⁺ cells were depleted by complement lysis from a population of immune splenocytes (isolated from mice immunized with SMAA complexes) in which class I-restricted CTL activity could be demonstrated in vitro (Fig. 2). Although untreated immune splenocytes were capable of clearing the virus infection, following depletion of CD8⁺ cells the remaining cells were unable to clear the virus infection. Furthermore, when equal numbers of immune splenocytes depleted of CD8⁺ cells were mixed with nonimmune splenocytes containing CD8⁺ cells prior to their adoptive transfer, this mixed population of cells also failed to clear the virus infection (Fig. 4). These results demonstrate that immunization with SMAA complexes induces CD8⁺ memory cells responsible for virus clearance and that these effector cells are not present in nonimmune mice and do not arise by stimulation with immune CD4⁺ helper cells following the adoptive transfer of splenocytes to X-irradiated mice infected with SV5.

Comparison of the immunogenicity of SMAA complexes (containing either *S. aureus* A or alum as the solid matrix) to infectious virus. In the majority of experiments involving SMAA complexes, we used fixed and killed suspensions of *S. aureus* A, which is not immunologically inert, as the solid matrix. Thus, the highly immunogenic nature of St. A-Ab-Ag complexes may, in part, be due to enhancement by protein A of the activation of the immune response. To evaluate the importance of the nature of the solid matrix to the immunogenicity of SMAA complexes, we constructed SMAA complexes that used an inert solid matrix as the basis of the complexes. Since alum has been licensed for use in humans and is a nonimmunogenic matrix, we developed a method for constructing Alum-Ab-Ag complexes (see Materials and Methods). Mice were immunized twice either with St. A-Ab-Ag (containing the HN and NP proteins) complexes,

FIG. 3. Comparison of the relative efficiency with which immune splenocytes that were or were not depleted of CD4⁺ or CD8⁺ cells enhanced the speed of clearance of SV5 infections in immunodeficient mice by day 10 postinfection. Lung extracts were prepared from infected immunodeficient mice that had adoptively received 2×10^7 splenocytes isolated from mice previously immunized with St. A-Ab complexes (tracks 1 to 4) or St. A-Ab-Ag complexes containing the HN, NP, P, and M proteins of SV5 (tracks 5 to 18). Prior to the adoptive transfer of the SV5-immune splenocytes, they were (tracks 9 to 18) or were not (tracks 5 to 8) sensitized for complement lysis and opsonization with MAbs to CD8 (tracks 9 to 13) or CD4 (tracks 14 to 18). Following complement lysis by anti-CD8 and anti-CD4 antibodies, the percentage of splenocytes that were CD8⁺ decreased from ~8 to 12% to <0.5%, and the percentage of CD4⁺ cells decreased from ~25 to 30% to <5%, as estimated by FACS analysis. The relative virus load in the infected lungs was estimated by measuring the amount of the P protein in lung extracts by Western blot analysis and autoradiography.

FIG. 4. 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 mice that had adoptively received untreated nonimmune splenocytes (tracks 1 to 3), untreated immune splenocytes (isolated from mice immunized with St. A-Ab-Ag complexes containing the HN and NP proteins of SV5; tracks 4 to 7), or immune splenocytes that had been depleted of CD8⁺ cells (tracks 8 to 11). In addition, lung extracts were made from mice which had adoptively received both untreated nonimmune splenocytes and immune splenocytes that had been depleted of CD8⁺ cells by complement lysis (tracks 12 to 15). Animals received a total of 3×10^7 splenocytes, with the exception of those represented by tracks 12 to 15; these animals received 3×10^7 nonimmune and 3×10^7 CD8-depleted immune splenocytes in a single injection.

FIG. 5. SDS-PAGE analysis (Coomassie brilliant blue-stained gel) of the purification of the HN and NP proteins of SV5 from soluble antigen extracts of infected cells either by St. A-Ab complexes (tracks 2 and 3) or by alum-Ab complexes (tracks 1 and 4). Polypeptides were separated by electrophoresis through a 12% SDS-polyacrylamide minislab gel. The positions of the SV5 polypeptides (HN and NP) and the antibody heavy (IgH) and light (IgL) chains are shown.

alum-Ab-Ag complexes (containing the HN and NP proteins; Fig. 5), or infectious virus. The level of neutralizing antibody in the sera of these mice was measured (Table 1).

Mice immunized with either St. A-Ab-HN/NP complexes or alum-Ab-HN/NP complexes had higher levels of neutralizing activity than animals immunized by prior infection with SV5. Indeed, the highest titers of serum neutralizing antibody were detected in mice immunized with alum-Ab-HN/NP complexes (Table 1). These animals were then killed, and different numbers of splenocytes were adoptively transferred to immunodeficient mice that had been infected with SV5. The amount of virus in these mice was estimated 10 days postinfection by Western blot analysis with a pool of MAbs specific for the P protein (Fig. 6). These results clearly demonstrate that splenocytes isolated from mice immunized with alum-Ab-HN/NP complexes were capable of clearing the infection. Furthermore, depletion of CD8⁺ cells from mice immunized with alum-Ab-Ag complexes abolished the ability of splenocytes isolated from these animals to clear the virus infection (data not shown). However, immunization with infectious virus was more efficient in inducing effector cells responsible for virus clearance than immunization with either St. A-Ab-Ag complexes or alum-Ab-Ag complexes. Thus, transfer of 1.6×10^6 splenocytes from mice immunized with infectious virus cleared the infection in 50% of the animals tested (in the absence of serum neutralizing antibody; Table 2), but the same number of splenocytes isolated from mice immunized with SMAA complexes failed to clear the infection in all animals (Fig. 6). This was despite the fact that mice which had received splenocytes from animals

TABLE 1. Titer of serum neutralizing antibody in immunocompetent mice immunized by prior infection with SV5, with a mixture of St. A-Ab-Ag complexes, or a mixture of alum-Ab-Ag complexes^a

Mouse no.	Neutralizing antibody titer after immunization with:		
	SV5	St. A-Ab-HN/NP complexes	Alum-Ab-HN/NP complexes
1	640	1,280	10,240
2	320	640	1,280
3	1,280	2,560	5,120

^a Different numbers of splenocytes isolated from these mice were adoptively transferred to immunodeficient mice, and their ability to clear virus by 10 days postinfection was measured (Fig. 6), as was their titer of serum neutralizing antibody (Table 2).

FIG. 6. Comparison of the relative abilities of splenocytes isolated from mice immunized with infectious virus (b), St. A-Ab-Ag complexes (containing the HN and NP proteins) (c), or alum-Ab-Ag complexes (containing the HN and NP proteins) (d) to enhance the speed of clearance of SV5 from the lungs of immunodeficient mice by day 10 postinfection. Lung extracts were prepared from infected immunodeficient mice (a) or from infected immunodeficient mice that had adoptively received either 4×10^7 , 8×10^6 , or 1.6×10^6 immune splenocytes. The relative virus load was estimated by detecting the P protein in lung extracts by Western blot analysis and autoradiography.

immunized with alum-Ab-HN/NP complexes had the highest titers of serum neutralizing antibodies (Table 2).

DISCUSSION

We have previously shown that SV5 establishes a persistent infection in mouse lungs and that neutralizing antibodies

TABLE 2. Titer of serum neutralizing antibody in immunodeficient mice^a

No. of lymphocytes transferred	Mouse no.	Neutralizing antibody titer in immunodeficient mice receiving splenocytes isolated from mice immunized with:		
		SV5	St. A-Ab-HN/NP complexes	Alum-Ab-HN/NP complexes
4×10^7	1	160	160	1,280
	2	80	320	<20
	3	320	160	320
	4	160	160	640
8×10^6	5	40	80	<20
	6	<20	<20	80
	7	80	<20	<20
	8	<20	<20	40
1.6×10^6	9	<20	<20	40
	10	<20	<20	<20
	11	<20	<20	40
	12	<20	<20	20

^a At 10 days after infection with SV5, immunodeficient mice adoptively received 4×10^7 , 8×10^6 , or 1.6×10^6 lymphocytes isolated from mice immunized by prior infection with SV5, with a mixture of St. A-Ab-Ag complexes (HN and NP), or a mixture of alum-Ab-Ag (HN and NP) complexes. The level of infectious virus present in the lungs of these mice was also estimated by Western blot analysis, and the results from this analysis are shown in Fig. 6. The mouse numbers given in this table correspond to the mouse numbers given in Fig. 6.

play only a minor role in clearing such infections (38). This conclusion is supported by the results presented here, which showed that splenocytes isolated from mice immunized with SMAA complexes containing the NP protein also cleared persistent SV5 infections of mouse lungs in the absence of serum neutralizing antibody. As was shown to be the case for immunization with infectious virus (38), the effector cells present in splenocytes isolated from mice immunized with SMAA complexes primarily responsible for virus clearance were CD8⁺ T cells. Although there is some debate as to how such cells may mediate their antiviral activity *in vivo* (i.e., directly through their cytolytic activity or through the release of antiviral lymphokines), the demonstration that immunization with SMAA complexes induced class I-restricted CTLs (as measured *in vitro*) suggests that these cells may be the CD8⁺ effector cells responsible for virus clearance *in vivo*. This premise is supported indirectly by the findings that immunization of mice with SMAA complexes was less efficient in inducing class I-restricted CTLs than immunization with infectious virus and that splenocytes isolated from mice immunized with SMAA complexes were also less efficient in clearing SV5 infections than were splenocytes isolated from mice immunized with infectious virus. This was not because the SMAA complexes were generally less immunogenic than infectious virus, since mice immunized with SMAA complexes (which contained the HN protein of SV5) made higher levels of neutralizing antibody than mice immunized with infectious virus. However, with regard to the efficiency with which SMAA complexes induce T-cell responses, it should also be noted that mice were not immunized with mixtures of SMAA complexes which contained all the SV5 proteins made by infectious virus (e.g., SH and F). Consequently, if there are important target antigens for T cells on those virus proteins not incorporated into SMAA complexes, it is possible that higher levels of class I-restricted CTLs and CD8⁺ effector cells could have been induced by mixtures of SMAA complexes that contained all the proteins encoded by SV5.

Although CD8⁺ effector cells are primarily responsible for clearing SV5 infections in mice (38), it is clear that in other virus infections serum neutralizing antibodies are protective. Indeed, to produce successful vaccines against a number of virus diseases, it may be important to induce both specific antibody and T-cell responses. While there are many potential advantages in producing noninfectious subunit vaccines, there also a number of problems to be overcome (25). One problem was thought to be that exogenous antigen could not induce class I-restricted T cells. We and others have now clearly demonstrated that this is not the case. However, the SMAA complexes used in this study are significantly less efficient in inducing class I-restricted T cells than infectious virus. By further understanding how exogenous antigen is processed for association with class I MHC antigens and by identifying the cell types involved in processing exogenous antigen for class I MHC presentation, it may be possible to devise more effective ways for inducing CD8⁺ cells with exogenous antigen, e.g., by targeting the binding of antigens to specific antigen-presenting cells or by directing antigens to particular intracellular processing pathways by influencing how they are taken up by antigen-presenting cells.

We have suggested that the manufacture of SMAA complexes may be one method for producing multivalent subunit vaccines (24). However, in the majority of our experiments on SMAA complexes, we used fixed and killed suspensions of *S. aureus* Cowan strain A as the solid matrix, which at present would not find favor for widespread use in humans.

Our initial results presented here demonstrate that alum-Ab-Ag complexes are as immunogenic as St. A-Ab-Ag complexes, both in their ability to induce antibody responses and in their ability to induce effector cells responsible for clearing SV5 infections from immunodeficient mice. These results therefore suggest that the immunogenicity of the solid matrix itself does not influence the immune response to the antigens of interest in the SMAA complexes. Rather, the presentation of antigens in a particulate form and possibly in repeating motifs may be of greater importance. In addition, it is possible that the presence of antibody bound to antigen may trigger immunological reactions (e.g., activation of the complement system) that will enhance the immune response to the SMAA complexes. Concerning the activation of T cells by SMAA complexes, the fact that the protein of interest is not bound directly to the solid matrix (or precipitated in it) may also be particularly advantageous. It can be speculated that proteins precipitated in alum or directly absorbed onto alum may not be processed correctly by antigen-presenting cells; this would explain why previous results have shown that antigens bound directly to alum have been poor inducers of T cells. While further work is required to answer these questions, it is clear that it should be possible to design inert solid matrices as carriers for antibody-antigen complexes for use in human vaccines, and indeed alum itself may prove suitable for this purpose.

ACKNOWLEDGMENTS

This work was supported by a grant from the Wellcome Trust. The Scottish Home and Health Department provided financial support for the purchase of the FACScan.

The photographic skills of Bill Blyth are gratefully acknowledged.

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