Cell-Mediated Immunity Induced in Mice after Vaccination with a Protease Activation Mutant, TR-2, of Sendai Virus

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Our previous study has shown that, although a trypsin-resistant mutant of Sendai virus, TR-2, replicates only in a single cycle in mouse lung with a negligible lesion, the animal acquires a strong immunity against lethal infection with wild-type Sendai virus, suggesting that TR-2 could be used as a new type of live vaccine (M. Tashiro and M. Homma, *J. Virol.* 53:228-234, 1985). In the present study, we investigated the immunological response elicited in TR-2-infected mice, particularly with respect to cell-mediated immunity. Analyses of cytotoxic activities of spleen cells with ⁵¹Cr release assays revealed that Sendai virus-specific T lymphocytes (CTL), in addition to natural killer activity and antiviral antibodies, were induced in DBA/2 and C3H/He mice infected intranasally with TR-2. Proteolytic activation of the fusion glycoprotein F was required for the primary induction of CTL, though not necessarily for stimulation of natural killer and antibody responses. Memory of the CTL induced by TR-2 was long-lasting and was recalled *in vivo* immediately after challenge with wild-type Sendai virus. In contrast to TR-2, immunization with inactive split vaccine failed to induce the CTL response, but it elicited a high titer of serum antibody and a low level of natural killer activity.

Sendai virus, a parainfluenza virus type 1, is prevalent worldwide as a contaminant in laboratory colonies of rodents (11, 31, 47). Under conventional conditions, most animals are affected by enzootic infections of the virus, which usually proceed subclinically in the upper respiratory tract. On the other hand, devastating acute epizootic lung infections often take place in mouse colonies, resulting in interruption of the maintenance and breeding of experimental animals. Control of Sendai virus infection is therefore a great practical problem. Although several attempts at vaccination with killed vaccines have been made (15, 21, 30, 47, 60), to date they are far from general use.

Sendai virus possesses two kinds of envelope glycoprotein, HANA and F (44). HANA mediates virus attachment to sialic acid-containing cellular receptors (49). The fusion glycoprotein, F, plays an essential role in virus entry; it mediates a membrane fusion of viral envelope with cellular membrane, and the genome RNA is introduced into host cells by this process (29, 49). F is synthesized in infected cells as a functionally inactive precursor and is proteolytically activated by trypsin or trypsinlike proteases into two disulfide-linked fragments, F₁ and F₂ (27, 29, 49-51). The proteolytic activation of F is therefore required for the virus to be infectious. Most tissue culture cell lines lack the activating protease; hence, the infection is limited to a single-cycle replication in these cells (27, 50, 54). On the other hand, if the activating protease exists in the hosts, e.g., chorioallantoic and amniotic cavities of embryonated chicken eggs (8, 45), certain primary culture cells (54), or mouse lungs (58), multiple cycles of replication are supported.

Sendai virus has exclusive pneumotropism in mice (11, 31,

57) despite wide distribution of the cellular receptors for the virus in various organs (34). Based on the cleavage-activation phenomenon, we have indicated that pneumotropism and pathogenicity of Sendai virus are primarily determined by the presence of an activating protease(s) in mouse lungs (28, 57, 58). The wild-type Sendai virus replicates in multiple fashion in the bronchial epithelial cells, which contain a trypsinlike protease(s) for activation of F, and causes a fatal lung lesion (58). We isolated a Sendai virus mutant, TR-2, in which F was resistant to both trypsin and the activator in mouse lungs but could be activated *in vitro* by chymotrypsin (33, 57). After activation with chymotrypsin, it can infect mouse lungs by a natural route of infection. However, the replication is limited to a single cycle and the lung lesion is only scanty, revealing that a chymotrypsinlike protease which activates TR-2 is absent in the lung (57). Nevertheless, the mice become immune and are strongly protected from challenge with wild-type Sendai virus (59). Considerable titers of hemagglutination-inhibiting (HI) antibody in the sera and neutralizing antibody of the immunoglobulin A (IgA) class in the bronchoalveolar lavages are found (59). These results indicated that TR-2 could be used as a novel type of live vaccine against Sendai virus infection (28, 59). We are therefore investigating the mechanism of immune protection induced in mice after single-step replication of TR-2 in the lung. In the present study, we examined cell-mediated immunity of mouse spleen cells after intranasal infection with TR-2.

MATERIALS AND METHODS

Wild-type Sendai virus. The Fushimi strain of Sendai virus was grown in 10-day-old chicken eggs and used as the wild-type virus. A mouse-adapted virus was obtained by serial passages of lung homogenates of mice infected intranasally with the wild-type virus. The adapted virus was 20 times as virulent for mice as the original wild-type virus and was used for the challenge experiments (59).

A trypsin-resistant mutant, TR-2. The procedures for

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isolation and characterization of a trypsin-resistant mutant of Sendai virus, TR-2, were described previously (33, 57). Active TR-2 was obtained by treating the LLC-MK₂ cell-grown virus with 25 µg of chymotrypsin (Sigma Chemical Co., St. Louis, Mo.) per ml (pH 7.2) for 10 min at 37°C. The enzyme action was then stopped by soybean trypsin inhibitor (Sigma) at 50 µg/ml (57).

Solubilization and UV irradiation of Sendai virus. Split Sendai virus was prepared by the method of Tsukui et al. (60). Egg-grown wild-type virus (2×10^6 hemagglutinating units [HAU]; 20 mg of protein) was solubilized in 0.125% Tween 80 in 100 mM Tris hydrochloride (pH 7.2), to which was then added an equal volume of diethyl ether (WAKO Pure Chemical Co., Osaka, Japan). After shaking the mixture vigorously, the water phase was collected and irradiated with a UV lamp at 3.5×10^{-5} J/mm² per s for 2 min. Formalin and glucose were then added to final concentrations of 0.2 and 5%, respectively, and the mixture was lyophilized. The preparation was suspended in saline before immunization. It expressed hemagglutinating activity (5×10^5 HAU/mg of protein) but lost infectivity to chicken eggs.

Animals. Specific-pathogen-free, 4- to 5-week-old male mice of strains C3H/HeNCrj (*H-2^k*) and DBA/2NCrj (*H-2^d*) were purchased from Charles River Japan, Inc., and kept under bio-clean conditions at 23°C and 55% humidity throughout the experiment.

Infection of animals. Mice were infected intranasally with 25 µl of TR-2 at 128 HAU/ml (3.8×10^6 PFU per mouse) under mild anesthesia with ether. Ten weeks later, the mice were challenged intranasally with 8.5×10^6 PFU of the mouse-adapted wild-type virus, which corresponded to 20 50% lethal doses (59). For immunization with split virus, 100 µg of the vaccine was given intraperitoneally. At intervals, mice were sacrificed and spleens were removed for the cytotoxicity test. In some experiments, 100 µl of a 10-fold-diluted IgG fraction of rabbit antiserum against purified neutral glycolipid ganglio-*N*-tetra-olylceramide (ASGM1) derived from bovine brain tissue, a kind gift from M. Naiki of the Hokkaido University Faculty of Veterinary Medicine, was intravenously injected 24 h prior to the cytotoxicity assay. Serum HI antibody was measured by the standard microtitration method (57).

Preparation of spleen cells. Single cell suspensions of spleen cells were obtained by grinding the spleens in a glass tissue homogenizer. Erythrocytes were removed by lysis with ACT buffer (140 mM NH₄Cl, 17 mM Tris hydrochloride, pH 7.2) at room temperature for 2 min. The cell suspension was washed three times and suspended in Iscove modified Dulbecco medium (GIBCO Laboratories, Chagrin Falls, Ohio) supplemented with 10% heat-inactivated fetal calf serum (FCS; Flow Laboratories, Inc., McLean, Va.). The number of viable cells was determined by trypan blue dye exclusion.

In vitro secondary stimulation of anti-Sendai virus cytotoxic T lymphocytes (CTL). For secondary in vitro stimulation of effector lymphocytes, 5×10^6 responder spleen cells from mice infected with TR-2 were placed in each well of 24-well tissue culture plates. Suspensions containing 10^5 syngeneic cells (L929 cells for C3H/He mice; P815 cells for DBA/2 mice) infected with wild-type Sendai virus for 12 h were added to each well as stimulator cells after X-ray irradiation at 2,000 R. Alternatively, UV-inactivated egg-grown wild-type viral particles (8 HAU per well) were added to each well. After incubation in Iscove modified Dulbecco medium containing 5×10^{-5} M 2-mercaptoethanol at 37°C for 5 days

in a CO₂ incubator, the viable lymphocytes were counted and used for the cytotoxicity test.

Antibody treatment of spleen cells. A spleen cell suspension at 10^7 cells per ml was incubated on ice for 45 min with anti-Thy-1.2 mouse monoclonal antibody of the IgM class (Serotec, Tokyo, Japan) at a dilution of 1:250, anti-Lyt-2.1 mouse monoclonal antibody of the IgG3 class (Cederlane Laboratory, Hornby, Ontario, Canada) at a dilution of 1:500, or rabbit immunoglobulins against purified ASGM1 at a dilution of 1:10. After being washed twice, the cells were incubated with a 1:15-diluted Low-Tox rabbit complement (Cederlane Laboratory) for 45 min at 37°C. The cells were washed twice and suspended in Iscove modified Dulbecco medium, and their viability was determined.

Preparation of target cells. The following three cell lines were used as target cells. The L929 cell line (*H-2^k*) is a fibroblast line derived from the liver of a C3H mouse and subcultured in minimum essential medium plus 10% bovine serum. YAC-1 cells (*H-2^d*), natural killer (NK)-sensitive cells derived from Molony murine leukemia virus-induced lymphoma in an A/Sn mouse, were maintained as a suspension culture in RPMI 1640 medium plus 10% FCS. P815 cells (*H-2^d*), NK-resistant cells, were derived from a methyl cholanthrene-induced mastocytoma of a DBA/2 mouse and maintained either in the same conditions as YAC-1 cells or in the peritoneal cavity of DBA/2 mice. For testing Sendai virus-specific CTL, L929 cells infected with wild-type Sendai virus at a multiplicity of infection of 5 PFU per cell for 12 h at 37°C were dispersed by 0.05% EDTA with or without 0.05% trypsin in phosphate-buffered saline (pH 7.2) and suspended in RPMI 1640 medium plus 10% FCS. P815 cells (5×10^6 /ml) were coated with UV-inactivated Sendai virus (128 HAU/ml) in the same medium at 37°C for 2 h and labeled with ⁵¹Cr. One milliliter of cell suspension in RPMI 1640 medium plus 10% FCS containing 100 µCi of sodium chromate (Na₂⁵¹CrO₄; The Radiochemical Centre, Amersham, Bucks, U.K.) was incubated for 2 h at 37°C in plastic flasks with occasional gentle shaking. The labeled cells were washed three times and suspended in RPMI 1640 medium plus 10% FCS to yield 2×10^5 cells per ml.

Cytotoxicity test. Samples (0.1 ml) of spleen cell suspensions (serial twofold dilutions from 1×10^7 to 2.5×10^6 cells per ml) were seeded in triplicate into the wells of 96-well half-area tissue culture plates (Costar, Cambridge, Mass.). To each well was added 50 µl of target cell suspension. The plates were incubated in a CO₂ incubator for 4 h at 37°C, and 0.1 ml of the supernatant was removed from each well for counting radioactivities in a gamma counter (ARC-351; Aloka, Tokyo, Japan). For measurement of spontaneous and maximum releases of ⁵¹Cr, 0.1 ml of Iscove modified Dulbecco medium and 1 N HCl were added, respectively. The percentage of ⁵¹Cr release was calculated according to the following formula: percent specific release = [(test release - spontaneous release)/(maximum release - spontaneous release)] × 100. Spontaneous releases of ⁵¹Cr against maximum release were as follows: uninfected L929 cells, 12%; Sendai virus-infected L929 cells, 23%; P815 cells, 14%; YAC-1 cells, 11%; Sendai virus-coated P815 cells, 21%. All data presented are the means of triplicate determinations.

RESULTS

Infection of C3H/He and DBA/2 mice with TR-2. When TR-2, which had been activated in vitro by chymotrypsin, was inoculated intranasally into C3H/He and DBA/2 mice, progeny virus in the lung was produced as an inactive form;

FIG. 1. Cytotoxic activities of spleen cells in mice after intranasal infection with chymotrypsin-activated TR-2. Male mice, 5 weeks old, of DBA/2 ($H-2^d$) and C3H/He ($H-2^k$) strains were infected intranasally with 25 μ l of TR-2 (128 HAU/ml) grown in LLC-MK₂ cells and activated in vitro by chymotrypsin. Spleen cell suspensions were prepared on the days indicated for testing NK activity against YAC-1 cells (■) and CTL activity against Sendai-virus-infected P815 ($H-2^d$) (●) or L929 ($H-2^k$) (○) cells by ^{51}Cr release assays. NK activity of control mice against YAC-1 cells (□) is included. Each plot indicates the mean of two to five mice, with bar indicating standard deviation.

hence, the infection terminated after single-cycle replication. As a result, lung lesions were barely induced and the mice remained healthy. Nevertheless, mice became immune from challenge with wild-type Sendai virus (data not shown), the results being comparable to those found previously in ICR mice (57, 59). Further experiments were carried out with 4- to 5-week-old C3H/He and DBA/2 mice.

NK activity of spleen cells after infection with TR-2. After intranasal infection of mice with TR-2, cytotoxic activity against YAC-1 cells appeared the next day and predominated until day 5 (Fig. 1). This activity was reduced drastically when the spleen cells were treated with anti-ASGM1 antibody and complement (Table 1). Furthermore, when the mice were injected intravenously with anti-ASGM1 IgG fraction 24 h prior to assay, cytotoxic activity was almost completely eliminated (Table 2). This treatment did not have any significant effect on virus titer in the lung and lung pathology (data not shown). These results showed that intranasal infection with TR-2 induced a transient NK activity in mice. It should also be noted in Fig. 1 that spontaneous NK activity of uninfected mice increased gradually from week 7 of life, whereas NK activity of TR-2-infected mice remained low after the transient appearance. The increase in NK activity in control animals could be explained by age dependency of NK activity in mice (32).

Sendai virus-specific CTL induced after infection with TR-2. After intranasal infection with activated TR-2, cytotoxic activity of spleen cells against Sendai virus antigen-

bearing target cells was detected between 5 and 14 days postinfection (Fig. 1). The activity was elicited only when the $H-2$ phenotype was compatible between mouse strain and target cells and was abolished by treating the spleen cells with anti-Thy-1.2 or anti-Lyt-2.1 antibody plus complement (Table 1). These results indicated that cytotoxic activity was due to CTL against Sendai virus. The CTL activity was partially suppressed by in vitro treatment with anti-ASGM1 plus complement (Table 1). This was consistent with an observation that part of the CTL population shares a common antigen marker of ASGM1 with NK cells (55). Sendai virus-infected L929 cells were equally sensitive to CTL irrespective of the activation state of F on their cell membranes, since CTL activity did not change even after treatment of the target cells with trypsin (data not shown).

In contrast to activated TR-2, when the same amount of inactive TR-2 was inoculated intranasally into mice, only a marginal CTL activity was elicited, although a transient NK activity and serum HI antibody response appeared (Fig. 2). The NK activity might be mediated by interferons which were stimulated by the noninfectious virus. These data showed that induction of CTL specific for wild-type Sendai virus was definite in mice after a single-step replication of TR-2 in the lungs and that inoculation of the activated virus was required for primary CTL induction in vivo.

In vitro secondary stimulation of CTL. Spleen cells, prepared from mice 8 to 10 weeks after infection with activated TR-2, were incubated in vitro for 5 days with either syngeneic stimulator cells bearing wild-type Sendai virus antigens or UV-irradiated viral particles. The former stimulator contained an uncleaved form of F, whereas the latter contained a cleaved form. Both stimulators could enhance Sendai virus-specific CTL activity (Fig. 3), indicating that memory of the CTL lasts for at least 8 to 10 weeks and that the presence of activated F was not essential for secondary induction of the CTL in vitro. Also note in Fig. 3 that the response of CTL to in vitro stimulation was three times lower in C3H ($H-2^k$) than DBA/2 ($H-2^d$) mice.

Cytotoxic activity of mouse spleen cells primed with TR-2 and challenged with wild-type Sendai virus. Ten weeks after the initial infection with TR-2, the mice were inoculated intranasally with 20 50% lethal doses of mouse-adapted Sendai virus. Neither virus replication nor pathological change was found in mouse lungs, and the mice remained healthy (data not shown). These results indicated that the C3H/He and DBA/2 strains infected with TR-2 acquired an immune status against wild-type Sendai virus, as has been observed previously with ICR strain animals (59).

In C3H/He mice, cytotoxic activity of spleen cells against YAC-1 cells increased a little just after the challenge infection and disappeared by day 5 (Fig. 4). Part of this activity was suppressed by treating the cells with either anti-ASGM1 or anti-Thy-1.2 antibody plus complement (Table 3). The precise nature of the activity was not known, though NK or lymphokine-activated killer cells could be suspected. In DBA/2 mice, this activity was barely detectable. In both mouse strains, a high level of Sendai virus-specific CTL activity, which was mostly eliminated by in vitro treatment with anti-Lyt-2.1 antibody and complement (Table 3), appeared without delay after the challenge infection (Fig. 4). The cytotoxic activity was kept at a higher level for a long period, at least for 2 weeks. This indicated that memory of Sendai virus-specific CTL, once generated by infection with TR-2, lasted longer than 10 weeks and was activated by the wild-type virus immediately after secondary infection by a natural route.

TABLE 1. Cytotoxic activities of mouse spleen cells after intranasal infection of activated TR-2

Mouse strain ^a	Days p.i. ^b	Spleen cells treated with ^c :	% Surviving spleen cells	Specific cytotoxic index (%) with given target cells at given E/T ratios									
				L929 (<i>H-2^k</i>)				P815 (<i>H-2^d</i>)				YAC-1 (<i>H-2^e</i>)	
				- Virus		+ Virus ^d		- Virus		+ Virus ^e		-Virus	
				100:1	50:1	100:1	50:1	100:1	50:1	100:1	50:1	100:1	50:1
C3H/He (<i>H-2^k</i>)	4	None	100	4.8	3.6	7.3	5.3	6.2	3.4	15.6	10.0	36.8	24.2
		Anti-ASGM1	68			2.3	2.5			4.3	4.1	8.8	6.5
		Anti-Thy-1.2	53			6.9	3.3			9.2	6.4	31.1	20.8
	11	None	100	8.4	3.3	26.0	22.1	10.4	6.1	8.7	4.4	6.8	3.6
		Anti-ASGM1	64			22.1	16.4			6.6	4.0	2.3	2.0
		Anti-Thy-1.2	49			11.6	8.6			2.3	2.2	5.1	4.6
		Anti-Lyt-2.1	56			10.9	8.1						
DBA/2 (<i>H-2^d</i>)	4	None	100	4.8	2.9	6.3	5.3	5.1	4.0	14.0	11.3	33.5	28.1
		Anti-ASGM1	70			2.1	3.0			4.4	2.0	4.6	2.1
		Anti-Thy-1.2	59			3.8	3.0			8.9	7.1	29.0	25.4
	12	None	100	6.9	5.5	8.1	6.6	11.4	4.6	42.2	35.1	4.8	2.0
		Anti-ASGM1	72							36.9	29.0	-0.3	0.8
		Anti-Thy-1.2	46							11.1	8.5	2.8	-0.4
		Anti-Lyt-2.1	51							12.9	9.6		

^a 5-week-old male mice.^b Intranasal inoculation with 25 μ l of chymotrypsin-activated TR-2 (128 HAU/ml). p.i., Postinfection.^c Spleen cells were treated with antiserum plus complements.^d L929 cells were infected with wild-type Sendai virus for 12 h.^e UV-irradiated egg-grown wild-type Sendai virus was incubated with the cells for 2 h at 37°C.

Cytotoxic activity of mouse spleen cells after immunization with split Sendai virus. Intraperitoneal inoculation of split Sendai virus was reported to induce serum HI antibody response and protection of mice from challenge with the virulent virus (60). It was therefore of interest to compare cellular immunity-inducing capacity between TR-2 and the split virus. When C3H/He mice were inoculated intraperitoneally with 100 μ g of the split virus, serum HI antibody to Sendai virus was detected at high titers (Fig. 5). NK activity was also detected for a few days (Fig. 5), although the activity was significantly lower than that in the case of TR-2 (Fig. 1). Specific CTL activity remained at a marginal level throughout the experimental period even when a booster immunization was done. A secondary in vitro stimulation by Sendai virus-infected L929 cells failed to elicit a significant increase in CTL activity (data not shown). When DBA/2 mice were used as hosts, the same results were obtained (data not shown).

DISCUSSION

The present study showed that Sendai virus-specific CTL as well as NK activity were elicited in mouse spleens after

intranasal infection with TR-2, which replicated only in a single cycle in bronchial epithelia and caused little pathological change in the lung (Fig. 1; Table 1). These mice were strongly protected from lethal challenge with wild-type Sendai virus (59), and memory of the CTL in mice lasted for >10 weeks and could be recalled immediately after challenge (Fig. 4). Since intranasal infection with wild-type Sendai virus causes severe pneumonia in mice which results in animal death as early as 10 days, it is difficult to investigate the Sendai virus-specific CTL induced after a natural route of infection. Therefore, most previous studies of CTL have been made by intraperitoneal inoculation of UV-inactivated virus or disrupted virus components. In contrast, TR-2 is nonpathogenic in mice, although it replicates in mouse lungs after intranasal inoculation (57). In this respect, TR-2 could be a valuable tool for investigation of cellular immunity induced by respiratory infection with Sendai virus.

Previous reports on Sendai virus-specific CTL have indicated that proteolytic activation of F glycoprotein is required for the primary induction of CTL (19, 20, 39, 42, 52), but replication of the inoculum virus is not necessary (19, 20, 52). The present results with TR-2 were principally consist-

TABLE 2. Effect of intravenous injection of anti-ASGM1 antibody on cytotoxic activities of spleen cells of C3H/He mice infected intranasally with TR-2

Days p.i. ^a	Mice treated with ^b :	Specific cytotoxic index (%) with given target cells at given E/T ratios					
		Sendai/L929 (<i>H-2^k</i>)		Sendai/P815 (<i>H-2^d</i>)		YAC-1 (<i>H-2^e</i>)	
		100:1	50:1	100:1	50:1	100:1	50:1
4	None	7.1	4.8	12.0	7.6	40.2	29.4
	Anti-ASGM1	5.5	3.3	5.9	2.3	8.8	4.0
14	None	21.3	16.8	3.7	4.6	9.0	7.7
	Anti-ASGM1	20.6	13.3	5.6	6.4	0.3	-0.2

^a 5-week-old male C3H/He mice were inoculated intranasally with 25 μ l of chymotrypsin-activated TR-2 (128 HAU/ml). p.i., Postinfection.^b Mice were injected intravenously with 0.1 ml of anti-ASGM1 antibody 24 h prior to cytotoxic assays.

FIG. 2. Cytotoxic activities of spleen cells and serum HI antibody of C3H/He mice after intranasal inoculation with inactive TR-2. Procedures were the same as those described in the legend to Fig. 1, except TR-2 was not activated by chymotrypsin treatment. Serum HI (Δ) antibody was determined by the standard microtitration method, using chicken erythrocytes. Symbols: $\blacksquare$, cytotoxic activity against YAC-1 cells; $\circ$, cytotoxic activity against Sendai virus-infected L929 cells.

ent with these observations. Inactive TR-2 possessing uncleaved F could hardly elicit CTL, although NK activity and serum antibody were stimulated (Fig. 2). UV-inactivated TR-2, treated by chymotrypsin to cleave F, did induce in vivo a low but definite CTL activity against Sendai virus (data not shown). However, the ether-treated split virus having activated F could not stimulate the specific CTL in vivo (Fig. 5). Therefore, it was suggested that incorporation of activated F into lipid bilayers of particles such as virion or liposome was necessary to induce primary CTL stimulation. Proteolytic activation of F was not essential, however, for secondary stimulation of the CTL both in vivo and in vitro (Fig. 4), as reported previously (20, 24, 42). Uncleaved F is known to function as a target molecule of CTL if it is integrated in the plasma membrane of target cells (2, 22, 37,

FIG. 4. Cytotoxic activities of spleen cells from TR-2-infected mice after challenge infection with wild-type Sendai virus. The procedures for infection of mice with TR-2 as well as for cytotoxicity assays were the same as those described in the legend to Fig. 1. On day 55 (DBA/2 mice) or 69 (C3H/He mice) after primary infection with TR-2, mice were infected intranasally with 20 50% lethal doses of mouse-adapted Sendai virus. On the indicated days after challenge infection, mouse spleen cells were tested for cytotoxic activities against YAC-1 cells ($\blacksquare$) or Sendai virus-infected P815 ($\bullet$) or L929 ($\circ$) cells.

FIG. 3. Secondary in vitro stimulation of Sendai virus-specific CTL. Infection procedures of mice with TR-2 were the same as those described in the legend to Fig. 1. Spleen cells, prepared from DBA/2 or C3H/He mice on day 69 or 51 postinfection, respectively, were incubated in vitro with either UV-inactivated wild-type Sendai virus (shaded column) or X-ray-irradiated Sendai virus-infected syngeneic cells, P815 cells for DBA/2 mice or L929 cells for C3H/He mice (white column). After incubation for 5 days at 37°C, viable lymphocytes were tested for Sendai virus-specific CTL activity against virus-infected P815 cells for DBA/2 mice or L929 cells for C3H mice by the ^{51}Cr release assay.

56). HANA glycoprotein is also suggested to be a principal target molecule (3, 23, 25, 37, 38). In our system, however, the role of HANA in either the induction of or the recognition by CTL remains to be elucidated.

It has been generalized that CTL require syngeneity of the major histocompatibility complex (*H-2*) of target cells (14). In Sendai virus-specific CTL, coincidence in the *H-2K* region seems important rather than that in the *H-2D* region (13, 25, 34, 35, 39, 41, 59). Several reports demonstrated that inbred mice of the *H-2^k* haplotype were low responders to secondary in vitro stimulation of CTL (14, 40, 53), whereas other investigations showed that *H-2^k* mice generated normal CTL responses (5, 7, 16, 24, 37, 39, 61). The present data indicated that CTL could be elicited in *H-2^k*-bearing C3H/He mice by intranasal infection with TR-2 and a secondary CTL response could be stimulated in vivo by reinfection with wild-type Sendai virus. This seemed to agree with the latter observations, although CTL response to secondary in vitro stimulation was apparently lower than that in DBA/2 (*H-2^d*) mice (Fig. 3). The reason for the different CTL responses in vivo and in vitro is not known.

NK activity was enhanced not only by intranasal inoculation of TR-2, but also by intraperitoneal inoculation of the split virus. Proteolytic activation of F was not required for this effect of the virus. Although NK cells were suggested to play an important role in elimination of Sendai virus from

TABLE 3. Cytotoxic activities of mouse spleen cells after challenge infection with wild-type Sendai virus

Mouse strain ^a	Days after challenge ^b	Spleen cells treated with ^c	% Surviving spleen cells	Specific cytotoxic index (%) with given target cells at given E/T ratios					
				Sendai/1929 (<i>H-2^k</i>)		Sendai/P815 (<i>H-2^d</i>)		YAC-1 (<i>H-2^a</i>)	
				100:1	50:1	100:1	50:1	100:1	50:1
C3H/He (<i>H-2^k</i>)	2	None	100	16.1	13.0	9.3	7.6	23.0	19.6
		Anti-ASGM1	67	14.3	12.2	8.8	4.4	12.5	6.9
		Anti-Thy-1.2	57	6.4	5.5	2.2	-1.0	18.8	16.1
		Anti-Lyt-2.1	61	6.8	4.9				
	7	None	100	24.0	20.0	9.6	8.1	2.4	5.8
		Anti-ASGM1	76	20.9	18.1	8.8	7.3	0.3	-0.1
		Anti-Thy-1.2	52	11.0	7.8	2.1	1.8		
		Anti-Lyt-2.1	64	8.3	6.6				
DBA/2 (<i>H-2^d</i>)	2	None	100	4.2	5.1	17.4	13.0	10.6	8.4
		Anti-ASGM1	71			14.1	9.8	5.3	-1.4
		Anti-Thy-1.2	48			5.4	4.0	8.1	6.6
		Anti-Lyt-2.1	57			4.9	-2.1		
	5	None	100			40.6	32.5	3.8	2.1
		Anti-ASGM1	62			35.2	28.6	-0.3	0.6
		Anti-Thy-1.2	53			10.3	7.7	1.0	0.9
		Anti-Lyt-2.1	56			8.8	5.7		

^a 5-week-old mice were infected intranasally with 25 μ l of chymotrypsin-activated TR-2 (128 HAU/ml) for 55 days for DBA/2 mice or 69 days for C3H/He mice.

^b TR-2-infected mice were challenged by intranasal infection with 20 50% lethal doses of wild-type Sendai virus.

^c Spleen cells were treated with antiserum plus complements.

infected mouse lungs (4, 7), no difference was found in either viral replication or development of pathological changes in the lungs of TR-2-infected mice before and after treatment with anti-ASGM1 antibody (data not shown). However, we could not exclude the role of NK cells in recovery from infection, since infection with TR-2 terminated after a single-cycle replication in the lung. CTL have been considered to be the most important factor for recovery from Sendai virus infection (6, 7, 17, 35), although serum antibodies (9, 10, 12, 26, 46) or interferons (4, 6, 18) may also be involved. In contrast, humoral immunity has been suggested to play a main role in protection from Sendai virus infection (15, 30, 41, 46, 60). Indeed, the mice immunized with the inactive split virus or inactive TR-2, which did not generate CTL but

raised serum antibody against Sendai virus (Fig. 2 and 5), were considerably protected from infection with the virus. Nevertheless, activated TR-2 could induce about 100 to 10,000 times stronger protection in several strains of mice tested so far than the inactive split virus and inactive TR-2 (unpublished data). Activated TR-2 can stimulate not only serum antibody but also neutralizing antibody of the IgA class in bronchoalveolar lavage (59), the latter of which was shown to play a major role in protection from Sendai virus infection (41). Although CTL is also suggested to play a role in protection (5), we do not know at present whether CTL induced by TR-2 infection is important in yielding strong protection. It is likely, however, that CTL minimizes the lung lesion by eliminating infected cells even if it could not inhibit the initial cycle of replication of incoming virus. From these aspects, as a live vaccine TR-2 has the advantage over inactive split virus. Inactive TR-2 or UV-light-irradiated inactive virus may be used as a secondary boosting antigen. Although immune protection from Sendai virus infection has been widely investigated, a consensus mechanism has not yet been determined. In considering vaccination against Sendai virus, the immunological factors essential for immune protection must be clarified. To determine this, adoptive transfer experiments with CTL clones as well as with monoclonal antibodies against Sendai virus are now in progress.

FIG. 5. Cytotoxic activities of spleen cells and serum HI antibody of C3H/He mice after intraperitoneal inoculation with split Sendai virus. The split virus was prepared by disrupting egg-grown wild-type Sendai virus with Tween 80 and ether as described previously (60). Male C3H/He mice, 5 weeks old, were inoculated intraperitoneally twice each with 100 μ g of the split virus (arrows). At variable intervals, mouse spleen cells were assayed for cytotoxicity against YAC-1 (■) or Sendai virus-infected L929 (○) cells by the ⁵¹Cr release assay. Serum HI antibody (▲) was determined by the standard microtitration method.

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