Journal of General Virology (1997), **78**, 1597–1603. Printed in Great Britain

Role of γδ TCR lymphocytes in the augmented resistance of trehalose 6,6′-dimycolate-treated mice to influenza virus infection

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Trehalose 6,6′-dimycolate (TDM), an immunomodulator, potentiates non-specific resistance in mice to influenza virus infection. When mice were injected intravenously with TDM, the striking proliferation of a minority of T-lymphocytes bearing gamma/delta T-cell receptors (γδ T-cells) that accumulated in granulomatous lungs was thought to be associated with the maintenance of acquired resistance to lethal influenza virus infection. To clarify the cellular basis of the defence against influenza virus, mice were depleted of γδ T-cells, alpha/beta (αβ) T-cells, or natural killer (NK) cells by *in vivo* administration of corresponding antibodies prior to influenza virus infection. The depletion of γδ T-cells significantly abrogated the augmented resistance of TDM-treated mice to infection, as did depletion of either αβ T-cells or NK cells. To gain insight into the functional ability of γδ T-cells, we evaluated the cytotoxic activity of this T-cell subset against a panel of target cell lines that were stably transfected with the influenza virus haemagglutinin (HA) gene from A/PR/8/34(H1N1) and A/Aichi/2/68(H3N2) strains. The γδ T-cells from TDM-treated mice showed profound cytotoxicity against the target cells expressing HA of either the H1 or H3 subtype, in a non-major histocompatibility complex-restricted manner. Taken together, these results indicate that γδ T-cells play a non-specific role, in conjunction with αβ T-cells and NK cells, in protecting mice against influenza virus infection, and that the recognition and destruction of HA-expressing target cells by the activated γδ T-cells is one of the steps involved in this anti-influenza virus immunosurveillance.

Introduction

A series of studies examining the proliferative response of T-lymphocytes bearing gamma/delta T-cell receptors (γδ T-cells) directed against a variety of infectious agents including bacteria, viruses and parasites has suggested that this T-cell subset plays a unique role in the early phase of host defence (Hiromatsu *et al.*, 1992; Nilssen *et al.*, 1996; Rosat *et al.*, 1993; Haas *et al.*, 1993). The *in vivo* use of specific anti-T-cell receptor (anti-TCR) antibodies and advances with TCR-knockout mouse models in experimental infections have further supported the view that γδ T-cells contribute to protective immunity, especially against bacterial and parasitic pathogens (Ladel *et al.*, 1995; Tsuji *et al.*, 1994). In mice infected with herpes simplex virus type 1 (HSV-1), γδ T-cells specifically recognize the viral glycoprotein I, which appears on the cell surface without the steps of antigen processing and presentation (Johnson *et al.*, 1992; Sciammas *et al.*, 1994). However, γδ T-cells have yet to be assigned any precisely defined functional role(s) in antiviral defence (Wallace *et al.*, 1995).

We have shown that mice inoculated intravenously with trehalose 6,6′-dimycolate (TDM), a glycolipid component of the mycobacterial cell wall, acquired high resistance to lethal influenza virus infection, involving the activation of macrophages and T-lymphocytes and resulting in earlier than normal interferon alpha/beta (αβ) production by these cells in response to the infection (Azuma *et al.*, 1987, 1988). We have also speculated that γδ T-cells that accumulate in the lungs of mice inoculated with TDM play a substantial role in the augmented

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resistance of these mice to influenza virus infection (Sazaki *et al.*, 1992). Reports by others, describing the prevalence of $\gamma\delta$ T-cells in the mouse lung during influenza virus (Allan *et al.*, 1992) or Sendai virus pneumonia (Ogasawara *et al.*, 1994) and noting the specific triggering of pulmonary $\gamma\delta$ T-cells by tuberculin-purified protein derivative (Augustin *et al.*, 1989) and the specificity of some $\gamma\delta$ T-cells for orthomyxovirus-infected cells (Ponniah *et al.*, 1996), have implicated $\gamma\delta$ T-cell involvement in anti-influenza virus immunosurveillance. The experimental model of TDM-treated mice, in which $\gamma\delta$ T-cells accumulated in the lung without influenza virus-specific stimulation, may be useful as an amplified system for analysing the functional role of $\gamma\delta$ T-cells in host defence mechanisms. Therefore, in this work, we have assessed the relative contribution of various cellular effectors *in vivo* to defence against influenza virus infection, and investigated the cytotoxic effector activity of TDM-elicited $\gamma\delta$ T-cells directed against targets expressing the influenza virus haemagglutinin (HA) gene.

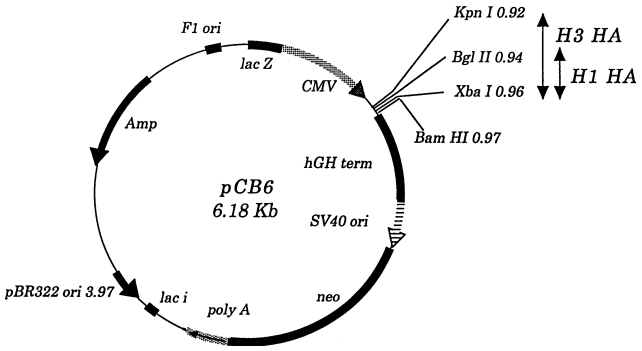

Fig. 1. Diagram showing the construction of HA-expressing plasmids pCB6HA1 and pCB6HA3. The full-length H1 HA or H3 HA gene was introduced into the indicated site of the adaptor plasmid pCB6, as described in Methods. Amp, ampicillin-resistance gene; neo, neomycin-resistance gene; CMV, immediate early promoter enhancer region of human cytomegalovirus; hGH, human growth hormone gene; SV40 ori, simian virus 40 enhancer early promoter element; F1 ori, bacteriophage f1 origin of DNA replication.

Methods

■ **Mice.** Female BALB/c mice were bred and maintained under specific pathogen-free conditions at the Animal Laboratory for Medical Research (Asahikawa Medical College) and were originally obtained from the Shizuoka Laboratory Animal Centre (Hamamatsu, Japan). Mice were used in experiments at 10–12 weeks of age. All animal manipulations were done at the above facility in accordance with institutional guidelines.

■ **Virus.** Mouse-adapted influenza virus A/PR/8/34(H1N1) was grown in the chorioallantoic sacs of 11-day-old chick embryos, following an initial passage in mouse lung, and stored in aliquots at −80 °C. The infectivity titre of the stock virus was $10^{5\cdot5}$ LD_{50} per 25 μl. Experimental infection was achieved by intranasally inoculating anaesthetized mice (pentobarbital sodium salt, 1 mg per mouse intraperitoneally) with 10 LD_{50} per 25 μl of the stock virus, and deaths were recorded during a 2-week-period.

■ **TDM treatment.** TDM prepared from *Mycobacterium tuberculosis* was purchased from Sigma. An oil-in-water emulsion of TDM (50 μg TDM in 200 μl emulsion) and a control emulsion were prepared as described by Azuma *et al.* (1987). Mice were inoculated intravenously with 200 μl of either TDM or control emulsion 10 days before virus infection.

■ **Construction of the HA expression vector.** In order to clone the HA cDNAs of influenza viruses A/PR/8/34(H1N1) and A/Aichi/2/68(H3N2), the following two pairs of oligonucleotides were synthesized using a DNA synthesizer model 380B (Applied Biosystems): TT38, 5′ CCTAGGATCCATGAAGGCAAACCTACTGG 3′ and TT39, 5′ AAGCTTCTAGATCTCAGATGCATATTCTGCACTG 3′; and GH1, 5′ CTGCAGTCTAGATAATACGACTCACTATAAGTAGAAACAAGGGTGTTTTT3′ and GH2, 5′ CTGCAGGGTACCAGCAAAAGCAGCGTCTAATTCTATTAATCATGAAG 3′. The cDNA of H1 HA was amplified by PCR with the primer-pair TT38/TT39 using pSPH1 as a template, whereas the cDNA of H3 HA was PCR-amplified with the primer-pair GH1/GH2 using a pool of cDNA that was prepared by reverse transcription with random primers using virion total RNA as the template. The TT38/TT39 PCR-amplified fragments were digested with *Bam*HI and *Xba*I and cloned into the *Bgl*II and *Xba*I sites of the expression vector pCB6, resulting in the H1-expressing vector pCB6HA1 (Fig. 1). The GH1/GH2 PCR-amplified fragments were digested with *Xba*I and *Kpn*I and cloned into the *Xba*I and *Kpn*I sites of the pUC119 vector (Takara Shuzo Co.). An excised insert, the sequence of which was confirmed, was then ligated into the *Xba*I and *Kpn*I sites of the expression vector pCB6, resulting in the H3-expressing vector pCB6HA3 (Fig. 1). Both constructs were sequenced to verify HA insertions and had complete identity with the published sequences. The eukaryotic expression vector pCB6 (obtained from Dr M. Roth, University of Texas, Tex., USA, with permission from Dr M. Stinski, University of Iowa, Iowa, USA) contains the immediate early promoter region of human cytomegalovirus and a gene encoding neomycin-resistance (Andersson *et al.*, 1989).

■ **Cells and transfection.** P815 cells (mastocytoma cells of DBA/2 mouse, H-2^d) provided by Dr Yasuhiro Hosaka (Osaka Pharmaceutical University, Osaka, Japan), and B16-4A5 cells (B16 melanoma 4A5 cells of C57BL/6 mouse, H-2^b) obtained from the Riken Cell Bank (Wako, Japan) were grown in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS) at 37 °C in a humidified 5% CO_2 atmosphere.

Transfection with pCB6HA1 or pCB6HA3 was achieved by a standard cationic liposome-mediated DNA transfer protocol (Felgner & Holm, 1989). Briefly, exponential phase cells were plated in 6-well plastic plates (Nunc) to 80% confluence 18 h prior to transfection. Cells were supplied with fresh growth medium 3 h before transfection, and the monolayer was washed three times with Opti-MEM I reduced-serum medium (Gibco BRL). Each well was overlaid with 400 μl of transfection cocktail containing 5 μg of plasmid DNA and 4% Lipofectin reagent (Life Technologies) in Opti-MEM I. Two days after transfection, transfected P815 and B16-4A5 cells were placed in selective growth media supplemented with 1 mg/ml and 2 mg/ml, respectively, of the neomycin analogue Geneticin (Sigma), and the cultivation was continued to allow outgrowth of drug-resistant clones. Neomycin-resistant colonies were pooled after 2 weeks, and stable transfectants, isolated by limited dilution, were screened by indirect immunofluorescence for high HA expression. Cells growing on 4-chamber slides (Nunc) were fixed with 3% freshly prepared paraformaldehyde in PBS at ambient temperature for 10 min. After extensive washing, the cells were incubated with a 1:25 dilution of anti-A/PR/8/34 rabbit serum (prepared by us) or anti-Aichi/2/68 chicken serum (a kind gift from Dr H. Kida, Hokkaido

University School of Veterinary Medicine, Sapporo, Japan), followed by incubation with an appropriate dilution of FITC-conjugated goat anti-rabbit IgG (E·Y Laboratories) or rabbit anti-chicken IgG (Sigma). Incubation was at room temperature for 1 h, followed by 3 × 5 min rinses in PBS. After immunostaining, the cells were mounted in 50% glycerol–PBS and analysed by confocal imaging of serial optical sections of 0·5 μm thickness on a Bio-Rad MRC600 laser scanning microscope. Fluorescent images were photographed from the microscope video-monitor. Selected clones stably expressing HA on the cell surface were designated K15.Y, clones expressing H1 and H-2^d; SI5, clones expressing H3 and H-2^d; MK46, clones expressing H1 and H-2^b; and AM10, clones expressing H3 and H-2^b. The clones were maintained thereafter in medium containing 400 μg/ml of neomycin.

■ **Isolation of effector lymphocytes.** Mice were killed by cervical dislocation 10 days after TDM or control emulsion treatment. The lungs were excised, cleared of extraneous tissues, washed three times and minced with scissors. Total lung cells were harvested in growth medium by gently straining the minced tissues through a stainless steel screen. The cell suspension was then layered onto Lympholyte M density gradients (Cedarlane) and centrifuged at 400 ***g*** for 20 min. Viable lymphocytes at the interface were collected and macrophages were depleted by adherence to plastic dishes. The non-adherent cell suspension was passed over a Cellect mouse T-cell enrichment immunocolumn (Biotex Laboratories). NK cells and αβ T-cells were depleted by using a magnetic cell separation device (Advanced Magnetics) with anti-asialo GM1 antibody (Wako) and goat anti-rabbit IgG-conjugated magnetic beads (PerSeptive Diagnostics), and anti-αβ TCR monoclonal antibody (Pharmingen) and goat anti-hamster IgG-conjugated magnetic beads, respectively. The purity of the resulting population typically ranged between 86% and 93%.

■ **Cytotoxicity assay.** The basis for the MTS/PMS-based (see below) colorimetric determination of cytotoxicity was adopted from Goodwin *et al.* (1995). Briefly, target HA transfectants and their parent non-transfected counterparts were seeded at a density of 10^3 cells/100 μl/well in 96-well flat-bottom microtitre plates (Nunc) with Ham's F10 medium supplemented with 10% FBS and incubated for 1 day at 37 °C. As effectors, lung γδ T-cells were then added at the indicated cell ratio (see below) to a total volume of 200 μl and admixed with the targets. After 24 h of co-cultivation, 40 μl of a reagent mixture containing 4·1 mM 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS, Promega) and 0·15 mM phenazine methosulphate (PMS, Sigma) was added to each well and incubated for a further 6 h at 37 °C. The optical densities (OD) were read on a micro ELISA reader (Labsystem Multiscan, Biochromatic) using a test wavelength of 492 nm and a reference wavelength of 520 nm. The percentage specific lysis (% SL) was calculated as follows: $\%\ SL = [(OD_T - OD_{BG}) - (OD_{T+E} - OD_E)/(OD_T - OD_{BG})] \times 100$, where T = target cells, E = effector cells and BG = background (medium only). In a preliminary experiment, the optimum E:T ratio was assessed at ratios of 5:1, 10:1 and 20:1. Greater killing was observed at 10:1 in comparison to 5:1, and no increased killing was observed at 20:1. Therefore, the E:T ratio of 10:1 was used for subsequent assays.

■ ***In vivo*-depletion of immune cell subsets.** The monoclonal antibodies (MAbs) used to deplete T-cell subpopulations *in vivo* were harvested from serum-free culture supernatants (GIT medium, Nippon Pharmaceutical Co.) of hybridoma lines (American Type Culture Collection, Rockville, USA) producing control hamster IgG MAb (UC8-1B9), anti-murine γδ TCR MAb (UC7-13D5) and anti-murine αβ TCR MAb (H57-597). The immunoglobulins of the supernatants were purified by ammonium sulphate precipitation and affinity chromatography on a protein A/G column (Pierce). The concentration of sterile-filtered preparations was measured using the Coomassie protein assay reagent. Mice were injected intraperitoneally with anti-TCR MAbs or control MAb (500 μg diluted in 0·3 ml PBS per mouse) on two occasions 2 days apart, starting 7 days after the TDM or control emulsion treatment. This schedule resulted in depletion of over 96% of the target T-cell population, as was judged by two-colour flow cytometry analysis of splenic lymphocytes from treated animals. For NK cell depletion, rabbit polyclonal anti-asialo GM1 serum (Wako) was used at a dose that efficiently suppressed the splenic NK cell-mediated cytolysis of YAC-1 target cells. Mice were injected intravenously with 50 μl of antibody (500 μg gamma globulin diluted in 0·3 ml PBS) on the day before infection with influenza virus. Control mice were injected with 500 μg of gamma globulin from non-immunized rabbits.

Results

Effect of NK depletion on the survival of TDM-treated mice infected with influenza virus

We have previously shown that NK cell activity markedly increased in TDM-treated and influenza virus-infected mice, suggesting that the NK cells participated in the resistance of these mice (Azuma *et al.*, 1987). Since NK cells are essential participants in the defence against most virus infections, we performed experiments *in vivo* to determine whether the inoculation of anti-asialo GM1 serum had any adverse effect on the acquired resistance of TDM-treated mice to lethal influenza virus infection. Fig. 2 shows that the acquired resistance of TDM-treated mice to infection was decreased by 60% in terms of survival when the mice were depleted of NK cells *in vivo*. In contrast, the control animals failed to combat this lethal infection whether they had been depleted of NK cells or not.

In vivo-depletion of γδ or αβ T-cells impairs the resistance of TDM-treated mice to infection with influenza virus

Our analysis favoured a role for T-lymphocytes in conferring high resistance upon TDM-treated mice to influenza virus infection (Azuma *et al.*, 1987, 1988). We therefore

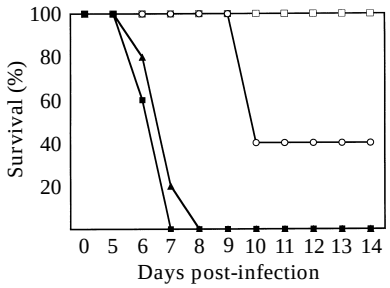

Fig. 2. Abrogation by anti-asialo GM1 antibody of the protective effect of TDM in mice exposed to influenza virus. Groups of BALB/c mice (n = 10), administered intravenously with 50 μg of TDM (□, ○) or control emulsion (■, ▲), were given either anti-asialo GM1 (■, ○) or isotype-matched control serum (□, ▲) on the day before infection with 10 LD_{50} influenza virus A/PR/8/34. Mouse survival was monitored daily for 14 days and plotted on the graph as a percentage of the control group.

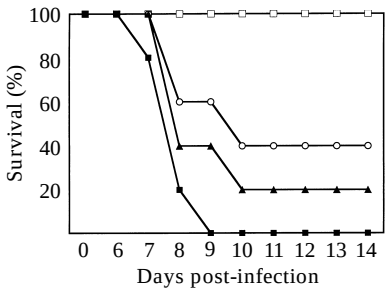

Fig. 3. Effect of T-cell subset *in vivo*-depletion on the survival of TDM-treated mice after influenza virus infection. Mice were injected with 50 μg of TDM (□, ○, ▲) or control emulsion (■). Groups of animals ($n = 10$) were then intraperitoneally inoculated with anti-$\gamma\delta$ TCR MAb (○), anti-$\alpha\beta$ TCR MAb (▲) or non-specific control hamster IgG (□, ■) 7 and 10 days after injection. Virus challenge was on day 11 with 10 LD_{50} of influenza virus A/PR/8/34. Mice survival was monitored daily for 14 days and plotted on the graph as a percentage of the control. Survival did not decrease in antibody-treated and mock-infected groups (data not shown).

investigated the relative contribution of T-cell subsets to this acquired resistance in mice depleted of $\gamma\delta$ or $\alpha\beta$ T-cells by the *in vivo* administration of anti-$\gamma\delta$ or anti-$\alpha\beta$ TCR MAbs, respectively. As shown in Fig. 3, the *in vivo*-depletion of either $\gamma\delta$ or $\alpha\beta$ T-cells abrogated the resistance of TDM-treated mice to influenza virus infection, resulting in 60% or 80% mortality, respectively, as compared with 100% survival of mock-infected animals. TDM-treated mice inoculated with control antibody did not show a decreased resistance to lethal infection, and no resistance was observed in mice treated with control emulsion and then inoculated with the control antibody. These results support the view that both $\gamma\delta$ and $\alpha\beta$ T-cells are necessary to confer protection upon TDM-treated mice against an influenza virus challenge.

TDM-elicited $\gamma\delta$ T-cells lyse HA-expressing target cells

The above results indicated that the $\gamma\delta$ T-cells played an important role in the defence mechanism. Since the HA spike protein of influenza virus is one of the main targets of immune recognition (Justewicz *et al.*, 1995; Johansson *et al.*, 1989; Simeckova-Rosenberg *et al.*, 1995), we examined whether or not target cells transfected with the HA gene could be recognized by effector $\gamma\delta$ T-cells. We established several transformed cell lines expressing HA H1 and HA H3 from the A/PR/8/34 and A/Aichi/2/68 strains, respectively. Fig. 4(*b*, *c*) shows the high levels of H1 HA and H3 HA on the cell surfaces of clones K15.Y and SI5, respectively, detected by indirect immunofluorescence microscopy, whereas no membrane fluorescence was found on the parental P815 cell line (Fig. 4*a*). The transfected clones shown in Fig. 4 (*e*, *f*) also bound chicken erythrocytes after differential trypsin treatment to activate the precursor HA to the mature form (Ellens *et al.*, 1990), suggesting that active HA is expressed on the cell surface. In addition to the H-2^d-transformed cell clones, the H-2^b lines, clones MK46 and AM10, expressing H1 HA and H3

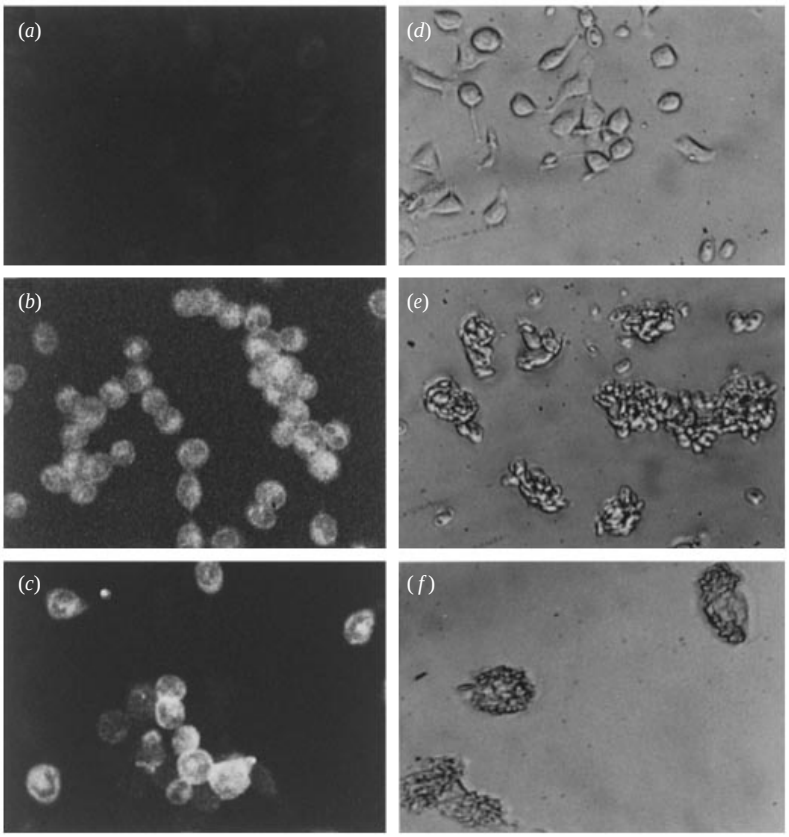

Fig. 4. HA expression on the surface of HA-gene-transfected P815 cells. P815 cells (*a* and *d*), H1 HA-gene-transfected cell clone K15.Y (*b* and *e*) and H3 HA-gene-transfected cell clone SI5 (*c* and *f*) were assessed for HA expression by indirect immunofluorescent staining (*a*–*c*) with isotype-matched control serum (*a*), anti-A/PR/8/34 polyclonal antibody (*b*) or anti-A/Aichi/2/68 polyclonal antibody (*c*), and FITC-conjugated secondary antibodies, and by haemadsorption for the ability to bind chicken erythrocytes on the cell surface (*d*–*f*). Original magnification × 200.

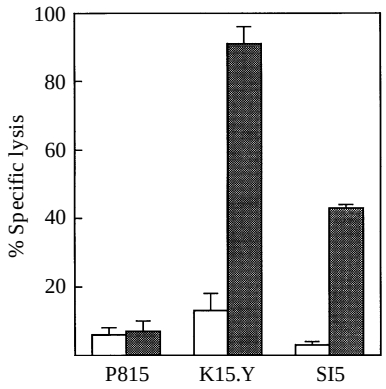

Fig. 5. Cytotoxic activity of TDM-activated γδ T-cells against HA-expressing cells. Cytotoxic effector activity of γδ T-cells from control mice (open columns) and TDM-treated mice (shaded columns) was determined by a 6 h microculture tetrazolium assay, see Methods. Fresh γδ T-cells were added to the MHC-matched targets, K15.Y and SI5, at an E:T ratio of 10:1. P815, parental cell line. Percentage specific lysis was calculated as described in Methods. Error bars shown are ± SD of triplicate values from three independent experiments.

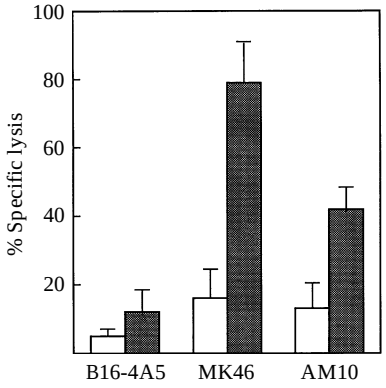

Fig. 6. MHC-unrestricted lysis of HA-expressing cells by TDM-activated γδ T-cells. The cytotoxic effector activity of γδ T-cells from control mice (open columns) and TDM-treated mice (shaded columns) against MHC-unmatched targets (H-2^b), MK46 and AM10 is shown. The assay system was the same as that for Fig. 5. Error bars are ± SD of triplicate values from three independent experiments.

HA, respectively, were also generated from the parental B16-4A5 cell line.

We investigated whether γδ T-cells from TDM-treated mice could lyse the HA transfectants. Fig. 5 shows a comparison of the cytotoxic activities of γδ T-cells from control and TDM-treated mouse lungs on H1 and H3 transfectants. TDM-elicited γδ T-cells exhibited a high level of cytotoxicity against both K15.Y and SI5 clones (91·4 ± 5·4% and 43·7 ± 0·8% lysis, respectively) when compared with that of non-treated control effectors (12·6 ± 4·5% and 3·3 ± 1·1% lysis, respectively). Despite the major histocompatibility complex (MHC) matching of target and effector cells as shown in Fig. 6, γδ T-cells from the lungs of TDM-treated mice markedly lysed both MK46 and AM10 clones (78·7 ± 10·8% and 40·4 ± 6·7% lysis, respectively) whereas control effector cells lysed the HA transfectants to a much lower extent (15·4 ± 7·8% and 12·9 ± 6·6% lysis, respectively). These results indicated that the cytotoxic effector activity of TDM-activated γδ T-cells directed against influenza virus HA is not MHC-restricted. Furthermore, to confirm that the cytotoxic activity was due to γδ T-cells and was independent of MHC, a cytotoxicity inhibition assay was carried out using anti-γδ TCR, anti-H-$2K^d$ and anti-H-$2K^b$ MAbs. Whereas marked inhibition of cytolysis by anti-γδ TCR MAb was observed, no inhibition of cytolysis by anti-H-$2K^d$ or anti-H-$2K^b$ MAbs was detected (data not shown).

Discussion

This report explores the role of γδ T-cells in the resistance of TDM-treated mice to lethal influenza virus infection. Our findings indicate an effector role for γδ T-cells that seems to be crucial in this antiviral immunosurveillance.

The finding that TDM-treated mice deficient in γδ T-cells could not fully overcome lethal influenza virus infection indicates that the remaining αβ T-cells could not maintain the optimum immune status necessary to confer high resistance to infection. In addition, in the αβ T-cell-depleted mice, the γδ T-cells failed to compensate for the deficiency in αβ T-cells, suggesting a complementary role for both T-cell subsets in the protection of mice primed against influenza virus infection. To further corroborate the above evidence, *in vivo* studies with TCR-knockout mice are in progress. Alternatively, in influenza virus infection, NK cells may be essential components of the cellular immune system (Skoner *et al.*, 1996; Stein-Streilein *et al.*, 1988). Our *in vivo* results from the NK-depletion system are consistent with this idea. In light of the recent demonstration that NK cells are reciprocally regulated by γδ T-cells in anti-bacterial host response (Ladel *et al.*, 1996), we suspect that the same phenomenon also occurs in anti-influenza virus immunosurveillance.

Although there is ample evidence concerning the triggering of lymphoid lytic machinery by γδ T-cells directed against a variety of target cells, little is known about this process in targets expressing virus components. We therefore assessed the cytolytic activity of TDM-activated pulmonary γδ T-cells towards targets expressing influenza virus HA. We found that the activated γδ T-cells mediated strong cytolysis of syngeneic targets (H-2^d) expressing either H1- or H3-type HA antigen. The same effectors also lysed the corresponding allogeneic targets (H-2^b), and cytolysis was not inhibited by H-2 specific MAbs, suggesting that this T-cell subset functions as an effector of MHC-unrestricted profiles of cell-mediated cytotoxicity. We confirmed that the lysis of transfectants was strongly inhibited by γδ TCR-specific MAb, suggesting an involvement of γδ TCR in the killing process (data not shown). The γδ T-cells killed the H1 HA-expressing cells (K15.Y and MK46) more efficiently than the cells expressing H3 HA (SI5 and AM10). At present the mechanisms by which the effector

cells recognize and kill the HA-expressing target cells are unknown and we cannot yet conclusively state that the effector cells are specific for HA of influenza virus. We have shown that target cells expressing H1 HA or H3 HA have an active form of HA on the cell surface that adsorbs chicken erythrocytes, suggesting that $\gamma\delta$ T-cells from TDM-treated mice recognize an unprocessed whole HA molecule. A HSV-1-specific $\gamma\delta$ T-cell clone, derived from the lymph node of an HSV-1-infected mouse, can recognize whole unprocessed HSV-1 glycoprotein gI in the absence of antigen-presenting cells (Johnson *et al.*, 1992; Sciammas *et al.*, 1994). *M. tuberculosis*-activated $\gamma\delta$ T-cells also express killer cell activity (Munk *et al.*, 1990). A similar activation mechanism might also be present in TDM-primed $\gamma\delta$ T-cells. The potent immunomodulatory cytokine IL-12 has been identified in tuberculous pleuritis (Zhang *et al.*, 1994). IL-12 induces lymphocyte subsets to mediate non-MHC-restricted cytotoxicity (Perussia *et al.*, 1992) and also enhances the expression of the granule mediators of cytotoxicity on a transformed $\gamma\delta$ T-cell line (Klein *et al.*, 1996). We postulate that TDM induces IL-12 in granulomatous lungs, which could have a potential effect in activating MHC-non-restricted cells at the inflammatory site. Further investigations are required to obtain a more comprehensive understanding of the mechanism(s) involved in influenza virus antigen recognition and target cell lysis by TDM-activated $\gamma\delta$ T-cells.

The overall conclusion from this study is that a concerted cellular immune response requiring the collaboration of T-cell subsets $\gamma\delta$ and $\alpha\beta$ and NK cells is necessary for the highly effective resistance to influenza virus infection shown by TDM-treated mice. Furthermore, the recognition and destruction of influenza virus HA-expressing target cells by activated $\gamma\delta$ T-cells is one of the essential steps in this antiviral immunosurveillance.

The authors would like to thank Dr Akira Ishihama (National Institute of Genetics, Mishima) for kindly providing the HA-expressing vector and Dr Hiroshi Kida (Hokkaido University School of Veterinary Medicine, Sapporo) and Dr Makoto Katagiri, (Asahikawa Medical College, Asahikawa) for kindly providing polyclonal antibodies to influenza virus and anti-H-2K MAbs, respectively. We acknowledge the very helpful support from Drs Masahiro Ogasawara and Yoshinori Takeda (Department of Microbiology) and Mr Kazuhiro Nakaya (Animal Laboratory for Medical Research, Asahikawa Medical College, Asahikawa) throughout the course of this study. Thanks also go to Ms Chiaki Hatanaka for secretarial support. This work was supported in part by a Doctoral fellowship to Md. M. Hoq from the Ministry for Education, Science and Culture, Japan, and was submitted for the PhD degree at Asahikawa Medical College.

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Received 29 November 1996; Accepted 20 February 1997