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Heterosubtypic Immunity to Lethal Influenza A Virus Infection Is Associated with Virus-Specific CD8^{+} Cytotoxic T Lymphocyte Responses Induced in Mucosa-Associated Tissues[1]

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Heterosubtypic immunity, defined as cross-reactive immune responses to influenza virus of a different serotype than the virus initially encountered, was investigated in association with virus-specific cytotoxic T lymphocyte (CTL) responses induced in systemic and mucosa-associated lymph nodes after immunization via different routes. Mice immunized by the pulmonary route with live nonpathogenic influenza virus, strain Udorn (H3N2), survived challenge with mouse-adapted pathogenic influenza virus, strain PR/8/34 (H1N1). These mice developed strong heterosubtypic CTL responses in spleen, cervical lymph nodes (CLN), and mediastinal lymph nodes (MLN). Alternately, only 20% of mice immunized intravenously, intraperitoneally, or intranasally survived the challenge; all of these developed CTL responses in spleen and CLN, but not in MLN. Direct correlation between short-term and long-term memory heterosubtypic CTL responses in MLN and host recovery after lethal infection indicates that these CTL responses may play an important role in heterosubtypic immunity. Furthermore, induction and maintenance of memory CTL in regional mucosa-associated lymphoid tissues are highly dependent on mucosal immunization. The results implicate the mechanism of heterosubtypic immunity and should be an important consideration in the development of protective mucosal vaccines against variant strains of influenza and HIV. © 1999 Academic Press

INTRODUCTION

Influenza virus undergoes periodic antigenic shifts in its two outer membrane glycoproteins, hemagglutinin and neuraminidase, thereby abrogating humoral cross-protection of different virus strains. However, cross-protection between different subtypes of influenza A virus is mediated by heterosubtypic immunity in the absence of virus-specific antibodies recognizing the outer membrane proteins (Schulman and Kilbourne, 1965). Heterosubtypic immunity to influenza virus infection is thought to be mediated by serotype cross-reactive cytotoxic T lymphocytes (CTLs) specific for the influenza nucleoprotein, a conserved gene product (Townsend *et al.,* 1984; Yewdell *et al.,* 1985; Taylor *et al.,* 1987; Wraith *et al.,* 1987). Passive transfer of large numbers of *in vitro* activated T cells possessing subtype-specific cytotoxic activity to influenza virus-infected mice can reduce pulmonary virus titers, promote their recovery, and provide protection in certain circumstances (Yap and Ada, 1978; Yap *et al.,* 1978; Lin and Askonas, 1981; Wells *et al.,* 1983; Lukacher *et al.,* 1984; Taylor and Askonas, 1986). However, it has been more difficult to induce resistance by active immu-

nization with shared antigens, such as NP and M proteins (Askonas *et al.,* 1982). Immunization with recombinant NP (Wraith *et al.,* 1987; Tite *et al.,* 1990), with NP expressed by poxvirus vectors (Andrew *et al.,* 1987; Andrew and Coupar, 1988; Webster *et al.,* 1991), or with purified M protein (Webster and Hinshaw, 1977) induced immune responses; however, subsequent protection upon challenge with homologous influenza virus is sometimes partial, but in other cases very weak or even absent. Immunization with recombinant vaccinia virus expressing an NP epitope recognized by CD8^{+} T cells readily induced primary pulmonary NP-specific CD8^{+} CTL. No protection by these T cells upon challenge with virus was observed, as determined by virus titers, clearance kinetics, and survival (Lawson *et al.,* 1994). *In vivo* depletion of CD8^{+} T cells by monoclonal antibodies led to partial but not complete reduction of hetersubtypic immunity (Liang *et al.,* 1994), and heterosubtypic immunity was observed in β_2-microglobulin-deficient mice (Eichelberger *et al.,* 1991; Bender *et al.,* 1994; Epstein *et al.,* 1997), raising questions regarding the effector mechanism of heterosubtypic immunity.

Mucosal immunization, i.e., total respiratory tract (TRT) exposure to live virus, has been a route for induction of heterosubtypic immunity to influenza since the early studies (Schulman and Kilbourne, 1965; Yetter *et al.,* 1980) and continues to be used (Liang *et al.,* 1994; Epstein *et al.,* 1997). Since influenza virus, like many other
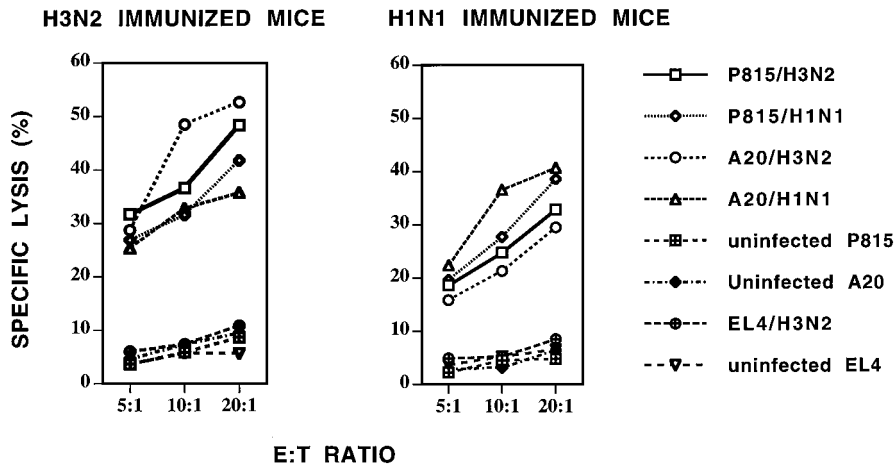
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FIG. 1. Induction of heterosubtypic CTL activity: BALB/c mice were infected intravenously with influenza virus type A, strains Udorn (H3N2) or PR/8/34 (H1N1) (three mice per group), and sacrificed 4 weeks later. Spleen cells were isolated, pooled, *in vitro* stimulated, and assayed for specific cytotoxic activity. Each effector cell culture was tested against H3N2 and H1N1 virus-infected ^{51}Cr-labeled MHC-matched P815 and A20 cells and MHC-mismatched EL-4 target cells.

pathogens, initiates infection at the mucosal surface of the respiratory tract, the immune response observed in systemic lymphoid organs may not reflect the events occurring at the site of infection, i.e., the mucosal surface. Immunizations through mucosal routes lead to induction of mucosal immune responses mediated by antigen-specific secretory IgA (S-IgA) antibodies at the site of immunization as well as at distant mucosal surfaces through the common mucosal immune system [see review by McGhee *et al.* (1987); Mestecky (1987)]. Detection of cell-mediated cytotoxicity in mucosa-associated tissues suggests that T-cell-mediated immunity is an important component for protection against mucosal pathogens (Davies and Parrott, 1981; Ernst *et al.,* 1985; Offit and Dudzik, 1989; Offit *et al.,* 1991). Mucosal immunization induces antigen-specific CTL responses predominantly in mucosa-associated lymphoid organs (Gallichan *et al.,* 1993), and maintenance of antiviral memory CTL in mucosal tissues is dependent on mucosal immunization as well as the time of assessment, as shown in studies with recombinant adenovirus vector (Gallichan and Rosenthal, 1996). However, it is not clear in these studies whether cell-mediated or humoral immune responses were critical for protection or recovery from infection. In addition, the duration of protective antigen-specific memory CTL responses in mucosa-associated tissues is not known.

An early study has reported that intraperitoneal immunization followed by TRT challenge with a heterologous serotype induced heterosubtypic CTL responses in mediastinal lymph nodes (MLN) (Bennink *et al.,* 1978); however, induction of CTL responses in various lymphoid compartments following immunization by different routes and their correlation with heterosubtypic immunity have not been systematically explored. In this study, we provide evidence that protective heterosubtypic immunity to

lethal influenza virus infection is correlated with heterosubtypic virus-specific CTL responses induced in mucosa-associated lymphoid tissues draining the site of infection. Furthermore, we demonstrate that mucosal immunization as opposed to systemic immunization generates long-lived protective heterosubtypic virus-specific CTL in a site-restricted fashion in mucosa-associated lymphoid organs. Heterosubtypic CTL responses induced in the mucosal compartment are discussed with regard to the effector mechanism of heterosubtypic immunity to lethal influenza A virus infection.

RESULTS

Induction of heterosubtypic CTL activity

To establish a model of heterosubtypic immunity, BALB/c mice were infected intravenously with influenza virus type A, either strain Udorn (serotype H3N2) or strain PR/8/34 (serotype H1N1). One month later the mice were sacrificed, and spleen cells were isolated, stimulated *in vitro,* and subjected to the virus-specific cytotoxicity assay. As shown in Fig. 1, heterosubtypic CTL activity to serotype H3N2 virus in mice immunized with serotype H1N1 virus was of comparable magnitude to homotypic CTL activity to the H1N1 virus at all E:T ratios tested. The CTL activities were measured by subtraction of nonspecific cytotoxic activity against mock-infected MHC-matched target cells (P815 cell line with H-2^d haplotype) and MHC-unrestricted cytotoxic activity against target cells with mismatched MHC haplotype (EL-4 cell line with haplotype H-2^b). Similarly, both homotypic CTL activity and strong heterosubtypic CTL activity specific for serotype H3N2 and H1N1 were detected in mice immunized with serotype H3N2. The cross-reactive CTL activities were observed when both the P815 cell line expressing MHC class I and A20 cells expressing MHC
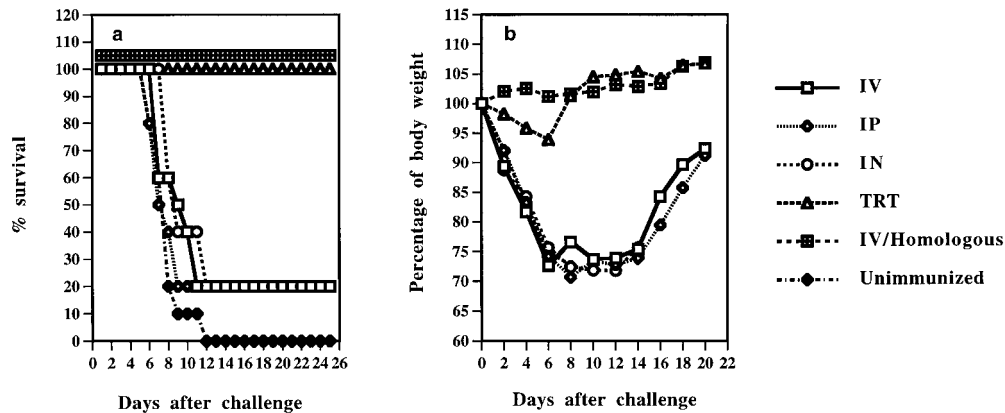
FIG. 2. Survival and loss of body weight in animals challenged with mouse-adapted heterologous influenza virus: BALB/c mice were immunized with influenza virus type A Udorn (H3N2) by different routes, as indicated. Four weeks later they were challenged with the heterologous mouse-adapted PR/8/34 strain (H1N1) via the TRT. Mortality was monitored daily for at least 3 weeks (a) and body weight of individual animals was measured every 2 days until all animals regained their initial weight (b). Values are means for 10 mice in each group.

class I and class II were used as target cells. Cytotoxic activities against the uninfected MHC-matched target cell line P815 (H-2^d) and the infected MHC-mismatched target EL-4 (H-2^b) cells were used as controls in all experiments. The results clearly showed that mice infected previously with the nonpathogenic serotype H3N2 strain Udorn developed strong homotypic and heterosubtypic CTL activity specific for the serotype H3N2 strain and the serotype H1N1 strain PR/8/34, respectively.

Survival of previously immunized mice following challenge with mouse-adapted serotype H1N1

Heterosubtypic CTL activity to serotype H1N1 influenza virus can be induced by immunization with serotype H3N2, and this serves as an excellent model for studying the role of virus-specific CTL in the host following infection. First, we induced heterosubtypic CTL responses specific for influenza type A using various routes of immunization with nonpathogenic influenza virus strain Udorn (H3N2). Even with this nonpathogenic virus strain, the mice immunized throughout the respiratory tract exhibited approximately 5% weight loss during the first 5 days but regained the weight by day 9. The other groups of animals did not show any signs of sickness (data not shown). One month later, 15 mice were divided into subgroups of 5 and 10 mice; 5 mice were subjected to CTL assay (see below), another 10 mice were challenged with the pathogenic strain (mouse-adapted) PR/8/34 (H1N1) via the TRT (250 PFU equivalent to 5 LD$_{50}$) in 50 μL. As shown in Fig. 2a, only the group of mice immunized previously via the TRT with live Udorn virus recovered totally (100%), while in the groups of naive mice and mice infected by intravenous (iv), intraperitoneal (ip), or intranasal (i.n.) routes, survival ranged from 0 to 20%. Mice previously infected intravenously with homologous serotype H1N1 virus were protected from TRT challenge

at a lethal dose. No weight loss was seen in this group of mice and the protection from secondary infection is known to be mediated by preexisting antibody specific for homologous outer membrane glycoproteins. A transient weight loss was observed in the group of mice immunized via the TRT (Fig. 2b). During the first week after challenge, the other groups of heterosubtypically immunized and unimmunized mice had severe weight losses from day 2 to day 6, when mortality was first observed.

Distribution of heterosubtypic CTL activity in various lymphoid organs following immunization via different routes

Although virus-specific CTL responses were generated after infection by most of the immunization routes, only the group of animals immunized by the TRT route survived the challenge with the mouse-adapted PR/8/34 strain. In the majority of studies performed in the past, CTL responses were usually measured in the spleen. Therefore, we examined the distribution of CTL responses in spleen and other lymphoid organs after immunization by various routes and protection upon subsequent challenge. To this end, BALB/c mice were infected with nonpathogenic strain Udorn (H3N2) via iv, ip, or i.n. routes (10^7 PFU/10 μl per mouse while awake) or via the TRT (10^7 PFU/50 μl through the nose of an anesthetized mouse). Four weeks later, on the same day that survival was tested by challenge, lymphoid tissues from five mice from each group were assayed for CTL activity against serotype H1N1 virus-infected target cells after *in vitro* stimulation. All subgroups of mice had strong heterosubtypic CTL activity against serotype H1N1 virus-infected target cells in cells from spleen (Fig. 3a); i.e., immunizations via iv, i.n., and TRT routes induced strong heterosubtypic CTL activity of similar magnitude, while immunization via the ip route induced
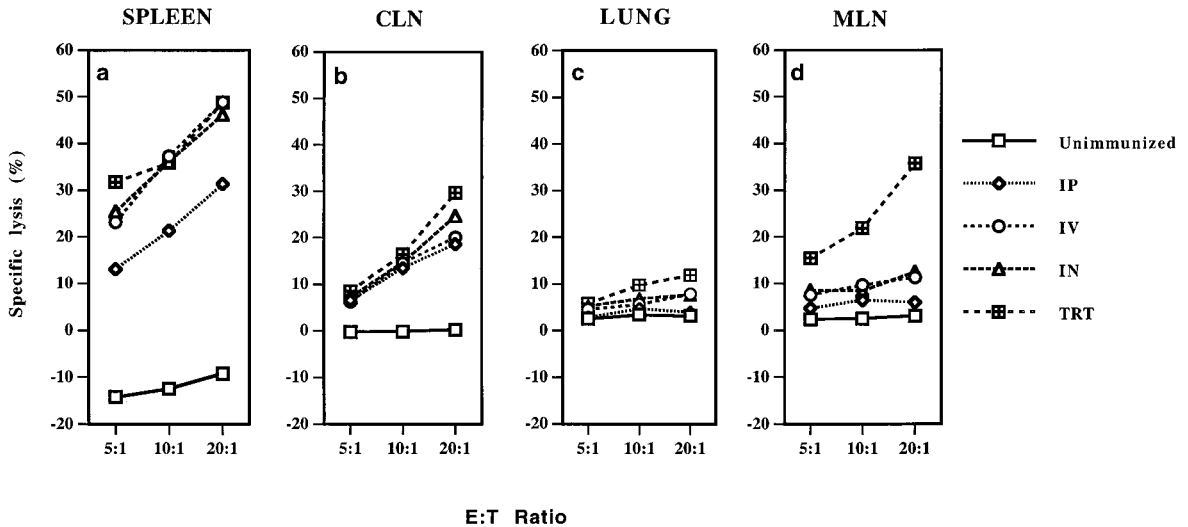
FIG. 3. Distribution of heterosubtypic CTL activity in different lymphoid organs following immunization by different routes: BALB/c mice were infected with nonpathogenic H3N2 strain Udorn by different routes, as indicated. Four weeks later, on the day of challenge, five mice from each subgroup were sacrificed. Lymphocytes isolated from different lymphoid organs were *in vitro* stimulated and assayed for CTL activity against H1N1 virus-infected P815 target cells. Specific CTL activities were determined by subtracting nonspecific cytotoxic activity against mock-infected P815 target cells.

somewhat lower heterosubtypic CTL activity. Also, heterosubtypic CTL activity was observed following *in vitro* stimulation of lymphocytes derived from CLN, but it did not differ significantly in mice immunized by the different routes (Fig. 3b). In contrast, stimulated lymphocytes isolated from lungs did not display CTL activity (Fig. 3c). Surprisingly, when lymphocytes isolated from MLN, the draining lymph nodes of the lungs were stimulated *in vitro* to measure CTL activity, positive heterosubtypic CTL activity was detected only in MLN of mice immunized via TRT. *In vitro* stimulated lymphocytes isolated from MLN of mice immunized through routes other than TRT remained unresponsive to virus-infected target cells (Fig. 3d). These data clearly showed that immunization with live nonpathogenic serotype H3N2 strain Udorn via TRT induced strong secondary heterosubtypic CTL activities against serotype H1N1 virus-infected target cells in both mucosal and systemic lymphoid organs, but not in the lungs.

Compartmentalized induction of virus-specific CTL responses and its correlation with survival rate

A comparison of the distribution of CTL responses in different lymphoid organs after immunization by various routes (Fig. 3), and the survival data after challenge (Fig. 2a), showed a correlation between the virus-specific CTL response induced in MLN and the recovery from infection (100% survival). On the other hand, the virus-specific CTL responses induced in other compartments did not correlate with recovery following challenge. The survival rates of the groups of animals immunized via iv, ip, and i.n. routes were similar (20% of survival).

Heterosubtypic CTL effector cells at the site of virus challenge

Since pneumonia is the cause of death after influenza infection, heterosubtypic virus-specific CTL at the site of infection may be essential for recovery. We reasoned that shortly after challenge, memory CTL precursors from MLN may migrate to the lung, the site of infection, and function in defense against the pathogen. Two days after challenge with mouse-adapted H1N1 influenza virus by the TRT route, lymphocytes isolated from lungs were assayed for virus-specific CTL activity without previous *in vitro* stimulation. Pulmonary lymphocytes of the mice immunized previously by TRT exhibited significant virus-specific CTL activity, while those obtained from animals immunized by other routes showed only marginal levels of specific lysis (Fig. 4a). When the effector cells were treated with monoclonal antibody specific for the CD8 molecule before the cytotoxic assay was performed, specific lysis was eliminated (Fig. 4b). This result indicated that heterosubtypic virus-specific CTL activity detected in the lung was mediated by CD8^{+} T cells.

Long-lived memory heterosubtypic CTL activity in mucosa-associated lymph nodes

To determine the longevity of heterosubtypic protection, 10 months after immunization with live virus Udorn (H3N2), lymphocytes isolated from MLN and spleens of mice immunized intravenously or through the TRT were assayed for CTL activity after *in vitro* stimulation. Heterosubtypic CTL activity against PR8/34 (H1N1)-infected target cells was detected in the spleen regardless of the route of immunization
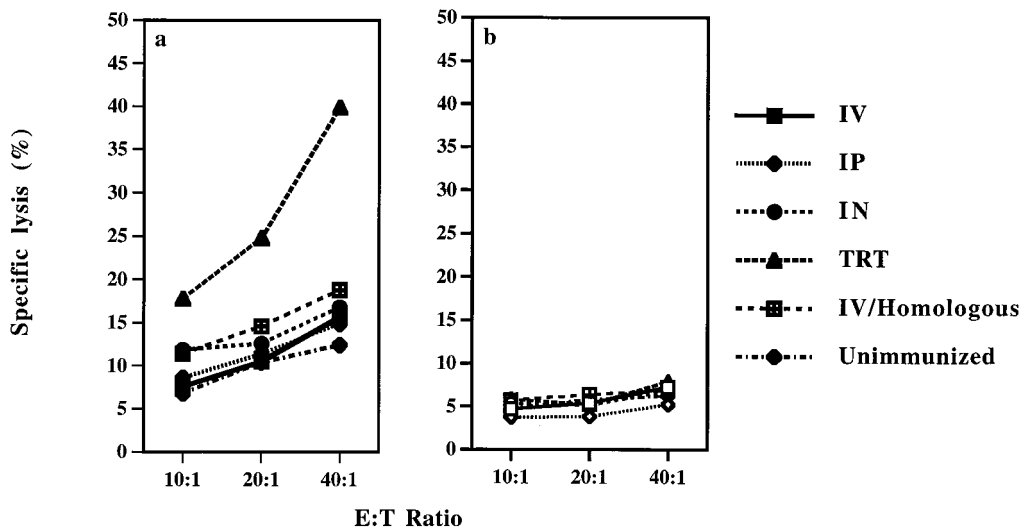
FIG. 4. CD8$^+$ cell-mediated specific CTL activity in the lung. Two days after challenging with mouse-adapted H1N1 influenza virus via the TRT, specific CTL activity of lymphocytes freshly isolated from the lung and without *in vitro* stimulation was assayed (a). Inhibition of specific CTL activity by pretreatment of effector cells with monoclonal antibody specific for CD8 (b).

(Fig. 5). However, heterosubtypic CTL activity was detected in MLN of mice immunized through the TRT but not in mice immunized intravenously. This activity in the MLN was approximately 70% of that detected in MLN of the mice 1 month after immunization. Again, an association between the long-lived memory heterosubtypic CTL induced at MLN and host recovery after challenge was maintained after 10 months (Fig. 6a). The percentage of animals surviving was slightly less than that of mice challenged 1 month after immuniza-

tion; 80% of TRT immunized mice survived challenge with a lethal dose of mouse-adapted strain, while no animal immunized intravenously survived this challenge. In addition, death occurred as early as day 7 after challenge, similar to the mortality of unimmunized mice (day 6). Signs of sickness were observed as early as 2 days after challenge, as measured by weight loss in these mice (Fig. 6b). All of the mice lost substantial body weight by day 14. Survivors started to recover after day 14, as evidenced by gain in body
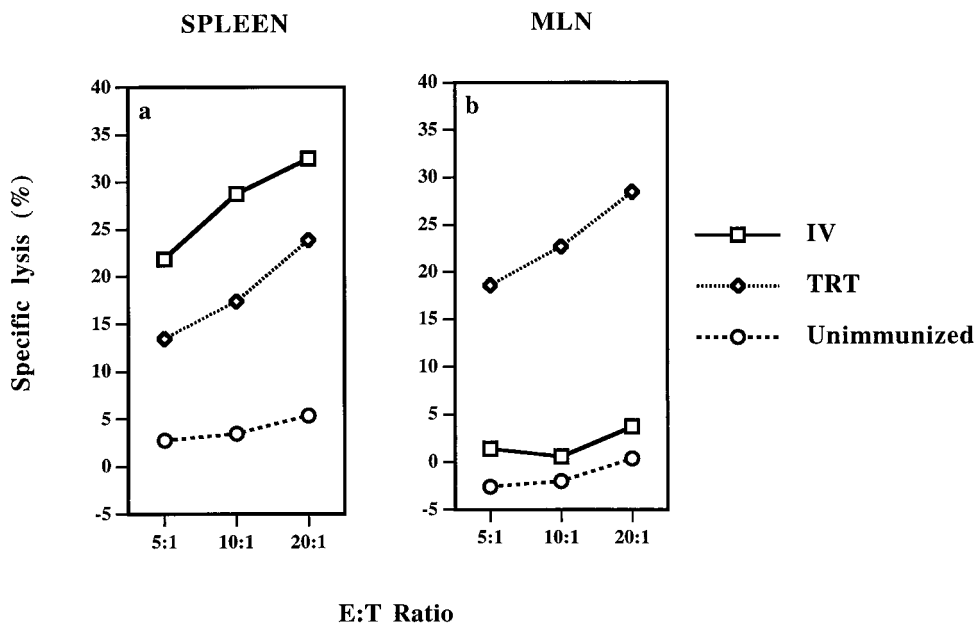

FIG. 5. Long-lived specific memory CTL responses in spleen (a) and MLN (b). BALB/c mice were infected with nonpathogenic H3N2 strain Udorn by different routes, as indicated; 10 months later, lymphocytes from MLN and spleen were stimulated and assayed for specific cytotoxic activity against PR8/34 (H1N1)-infected target cells.
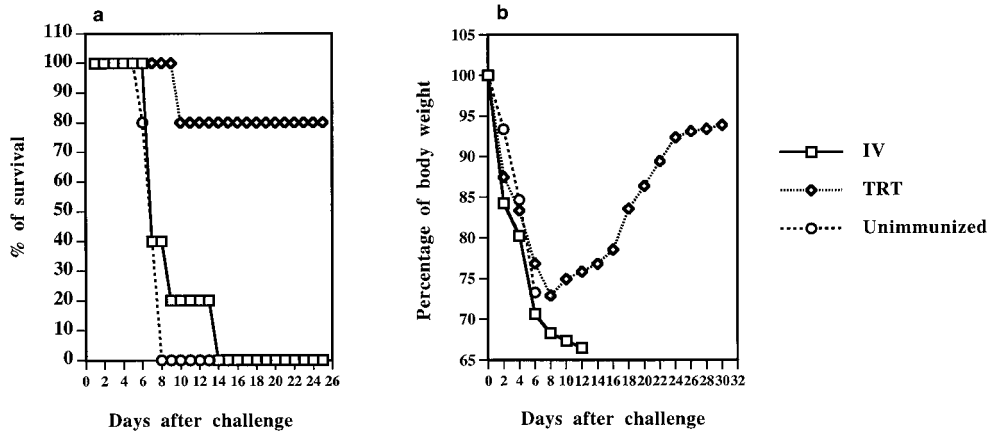
FIG. 6. Long-lasting heterosubtypic immunity protects from challenge with mouse-adapted heterologous virus: BALB/c mice were immunized with influenza virus type A Udorn (H3N2) by different routes, as indicated. Ten months later they were challenged with heterologous mouse-adapted PR/8/34 (H1N1) via the TRT. Mortality was monitored daily for at least 3 weeks (a). Body weight of individual animals was measured every 2 days until all animals regained their initial weight (b). Values are means for 10 mice in each group.

weight. The length of time needed to recover from challenge was longer in these mice challenged 1 month after immunization.

Serum antibody

Four weeks after immunization with influenza virus strain Udorn (H3N2), serum influenza-specific antibody titers were determined before challenge with mouse-adapted PR/8/34 (H1N1). The results are shown in Fig. 7. No statistically significant difference in serum influenza-specific antibody titers was found between groups of immunized mice. Therefore, no correlation between ELISA antibody titers and heterosubtypic immunity was observed.
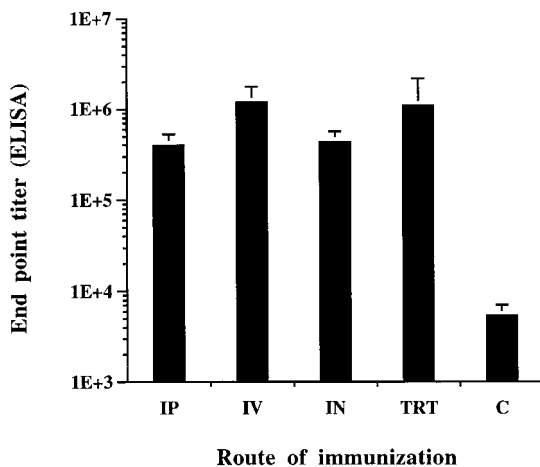

FIG. 7. Influenza-specific serum antibody titers. BALB/c mice were immunized with influenza virus type A Udorn (H3N2) by different routes, as indicated. Four weeks later, ELISA antibody titers were determined before challenge with the heterologous mouse-adapted PR/8/34 strain (H1N1) via the TRT. The values represent the mean and SD of the end-point ELISA antibody titer for five mice in each group.

DISCUSSION

We investigated the association between antigen-specific CTL responses induced in different lymphoid compartments and host protection in order to elucidate the effector mechanism of heterosubtypic immunity of influenza virus infection. Several important conclusions can be drawn from this study: (a) Heterosubtypic CTL responses induced in systemic lymphoid organs (spleen) failed to confer complete protection from lethal mucosal infection via TRT. (b) Antigen-specific CTL responses induced in mucosa-associated lymphoid tissues, draining the site of lethal infection, but not systemic lymphoid organs correlated with complete host protection after mucosal challenge. (c) Induction of antigen-specific CTL responses in mucosa-associated lymphoid tissues after mucosal immunization is highly region-restricted and does not follow the concept of a common mucosal immune system, as demonstrated for antibody responses. (d) Mucosal immunization induces long-lived antigen-specific CTL in systemic and mucosa-associated lymphoid tissues. In contrast, systemic immunization induces preferably long-lived antigen-specific CTL in systemic, but not mucosa-associated, lymphoid tissues.

BALB/c mice infected iv, ip, or i.n. with the nonpathogenic H3N2 strain Udorn developed high levels of heterosubtypic CTL activity as well as homotypic CTL responses mediated by CD8$^+$ influenza virus-specfic CTL in the spleen. However, the survival rate after challenge with mouse-adapted H1N1 strain PR/8/34 was low in all groups (20% survival). This result indicates that heterosubtypic CD8$^+$ influenza virus-specific CTL responses induced in a systemic immune compartment, such as the spleen, are not sufficient to protect the animals from death after mucosal lethal infection via TRT. Several studies demonstrated that heterosubtypic protection against distinct influenza serotypes in mice can be pro-

vided by passive transfer of polyclonal or monoclonal CTL specific for the shared NP antigen (Taylor and Askonas, 1986). However, the numbers of influenza-specific CTL used in these adoptive transfer studies were unphysiologically high and ranged from 3×10^6 cloned L4 Tc cells (Lin and Askonas, 1981), to 10^7 cloned CTL cells (Lukacher et al., 1984), and 10^8 splenocytes from immunized mice (Yap et al., 1978). Furthermore, induction of heterosubtypic CTL responses in spleen after active systemic immunization with shared protein antigens, such as the NP and M proteins, usually failed to induce complete protection from challenge (Askonas et al., 1982). In accordance with the latter, the results obtained in our study model indicate that antigen-specific CTL responses induced in systemic lymphoid organs by active immunization are not reflective of host protection after mucosal challenge with heterologous virus.

TRT exposure to live virus has been a route for induction of heterosubtypic immunity to influenza since the early studies (Schulman and Kilbourne, 1965; Yetter et al., 1980; Liang et al., 1994; Epstein et al., 1997). We found that heterosubtypic immunity could be protective only when mice were initially exposed to influenza virus through TRT, the route used for lethal challenge to test induction of protection. Mortality was chosen in our study to better demonstrate heterosubtypic immunity, since reduction of virus replication in the lung varies from one study (Liang et al., 1994) to another (Epstein et al., 1997) and heterosubtypically immune mice exhibit only modest reductions in lung virus titer, but significant heterosubtypic protection against mortality upon challenge compared with controls (Epstein et al., 1997). It is therefore likely that variations in virus titers measured in the lungs of mice in different experimental groups would not be significant and would not reflect heterosubtypic immunity as demonstrated by protection against mortality. In addition, the lethal dose of influenza virus (250 PFU or $5 \times LD_{50}$) was used to achieve 100% mortality in naive mice. This was the optimal dose in order to compare protection among the various groups. Mice immunized by TRT suffered weight loss following immunization and it is likely that this route of immunization resulted in virus replication at the mucosal surface and therefore increased the antigenic load in mucosa-associated lymphoid tissues. This result may be responsible for induction of a mucosa-related antigen-specific CTL response that is associated with protective heterosubtypic immunity to lethal influenza virus infection. Other initial infection routes, such as iv, ip, or i.n., failed to generate total recovery from heterosubtypic lethal influenza challenge, although all immunization routes developed a high level of virus-specific CTL activity in the spleen as well as influenza-specific antibody titers in serum. Our study indicates that CD8+ virus-specific CTL response induced in local mucosa-associated lymphoid organs is of paramount importance for successful protection. Indeed, we found a cor-

relation between protective heterosubtypic immunity and heterosubtypic CD8+ influenza virus-specific CTL responses induced in the MLN, the draining lymph nodes of the lower respiratory tract. In addition, CD8+ CTL were recovered from the lungs of mice infected previously through TRT 2 days after challenge, but not from mice immunized by other routes. Although the latter group of mice developed significant secondary heterosubtypic CTL activity in the spleen, this CTL activity did not correlate with protection. An early study reported induction of heterosubtypic CTL responses in MLN of mice after initial ip immunization followed by TRT challenge with a heterologous serotype (Bennink et al., 1978). In this study, CTL activity was detected in MLN on day 4, but not day 2, after challenge. TRT challenge could have enhanced the induction of CTL on day 4 in the MLN following the primary ip immunization, since primary CLT activity in MLN normally is detected on days 4, 5, and 6 after TRT challenge of naive mice (data not shown). In our study, the CTL responses following in vitro restimulation were assayed before the challenge, i.e., 30 days after the initial immunization with heterologous virus, in order to confirm induction of heterosubtypic CTL responses by primary immunization through different routes. It is interesting that immunization by TRT, but not other immunization routes, induced memory antigen-specific CTL responses in MLN that differentiated into CTL effectors, early after the challenge, i.e., on day 2, in the lungs. This CTL activity seems to play an important role in host recovery, since naive mice do not survive challenge, although they do develop CTL in MLN, but at least 2 days later. The involvement of T cells in heterosubtypic immunity to lethal influenza A virus infection is still under investigation. Previous T cell depletion studies contradicted each other, in that antigen-specific CTL in the lungs seem to control virus replication in one report (Liang et al., 1994), while in other investigations depletion of T cells had no significant effect on heterosubtypic immune protection and virus clearance in the lungs (Bender et al., 1994; Epstein et al., 1997). In our study, CD8+ CTL responses induced in MLN after TRT immunization correlated with host survival upon challenge.

Because mucosal immunization induced both primary and secondary virus-specific CTL responses in regional mucosa-associated lymphoid tissues, it can be hypothesized that shortly after challenge, virus-specific CTL memory cells in the regional lymph nodes become effector CTL and localize to the site of infection. Indeed, this concept is supported by recent studies with Sendai virus (Hou and Doherty, 1993) and by the kinetics of cytokine mRNA expression after influenza virus infection (Carding et al., 1993). Local immunity should be a more effective way to combat pathogens in the respiratory tract, since as many as 10^7 cloned CTL cells were needed for adoptive transfer to generate systemic het-

erosubtypic immunity able to clear influenza infection in the lungs (Lukacher et al., 1984).

With regard to the compartmentalization of the virus-specific CTL responses, our data suggest that the distribution of influenza-specific CTL responses in lymph nodes, associated with the mucosal surface of the respiratory tract, is highly dependent on the route of initial immunization. For example, immunization throughout the respiratory tract was the only route to induce a significant CTL response in MLN. On the other hand, CTL responses induced in spleen and cervical lymph nodes (CLN) were independent of immunization route. Furthermore, CTL effectors induced in CLN do not correlate with host survival and reflected CTL activity in the spleen, suggesting a strong link of CLN with the systemic compartment rather than the lower respiratory tract. CTL effectors detected in CLN presumably play a role in virus clearance in the upper respiratory tract based on their location. Induction of compartmentalized virus-specific CTL responses in mucosa-associated lymphoid tissues and their importance in immune protection have been reported previously (Gallichan et al., 1993). Although protective immunity was induced following i.n. immunization, this i.n. immunization protocol very likely involved the entire respiratory tract based on the relatively large volume of inoculum given to anesthetized mice. Our immunization protocol distinguishes between the i.n. immunization route and the TRT and demonstrates that induction of protective antiviral memory CTL in mucosal tissues is dependent on site-specific immunization. Thus, cell-mediated immune responses seem to play an important role in mucosal immune protection. Mucosal immunity has previously been discussed almost exclusively with regard to humoral immunity (McDermott and Bienenstock, 1979; Mestecky, 1987; McGhee et al., 1992; Mestecky and Jackson, 1994; Russell et al., 1999). In contrast to protective mucosal humoral immunity, virus-specific CTL responses after mucosal immunization are restricted to the immunized region (draining lymph nodes), since initial i.n. and intravaginal immunization did not induce CTL responses in MLN or confer protection against heterologous virus challenge (data not shown).

Finally, the effect of initial immunization on compartmentalized induction of memory heterosubtypic influenza virus-specific CTL responses, and their role in recovery after lethal infection with heterosubtypic virus, was best demonstrated by challenge experiments 10 months after initial immunizations. Long-lasting memory CTL responses were observed in systemic lymphoid organs, such as spleen, regardless of the route of initial immunization. However, the compartmentalized memory CTL response in the MLN was observed in animals only after immunization throughout the TRT. A recent study of recombinant adenovirus vector expressing glycoprotein B of herpes simplex virus (Gallichan and Rosenthal,

1996) has indicated that long-lived CTL responses compartmentalized into mucosa-associated lymphoid tissues (MLN) or systemic lymphoid organs (spleen) depending on the route of immunization, i.e., to mucosal sites following i.n. administration and to systemic sites following ip administration. Thus, no systemic antigen-specific CTL response was observed following i.n. immunization with recombinant adenovirus vector, while immunization with influenza virus by TRT induced antigen-specific CTL responses in mucosa-associated lymphoid tissue (MLN) as well as in the spleen, a systemic lymphoid organ. This distinct pattern of compartmentalization of long-lived virus-specific CTL responses to recombinant adenovirus might be due to different pathogenic characteristics of this virus vector, although no data have been reported to confirm this notion. Ten months after initial immunization, heterosubtypic CTL responses declined, resulting in prolonged sickness upon challenge prior to recovery. The CTL activity observed in the MLN was nevertheless associated with survival upon intrapulmonary challenge.

In conclusion, we demonstrated that CD8-mediated heterosubtypic virus-specific CTL response induced in mucosa-associated lymphoid tissues associated with the site of infection correlated with heterosubtypic immunity to lethal influenza virus infection. However, the question whether the CTL-mediated lysis of virus-infected cells is the only or primary effector mechanism in heterosubtypic immunity is still open for debate. The induction and maintenance of the heterosubtypic CTL response in mucosa-associated lymphoid tissues are highly dependent on the route of immunization. These results shed new light on the importance of cell-mediated heterosubtypic immunity at mucosal surfaces and should be an important consideration in the development of new protective mucosal vaccines against variant strains of influenza and HIV.

MATERIALS AND METHODS

Mice

BALB/c (H-2^d) mice were purchased at 6 weeks of age from Charles River Laboratories (Raleigh, NC). All mice were maintained in horizontal lamina flow cabinets and were provided sterile food and water ad libitum. The mice used in the experiments were between 8 and 10 weeks of age.

Viruses

Influenza virus strain A/Udorn/307/72 (H3N2) (a gift from Dr. B. Murphy, National Institutes of Health, Bethesda, MD) was grown in the allantoic cavity of 10-day-old embryonated hen eggs for 48 h at 35–36°C. The fluid was clarified by centrifugation at 4000 g for 20 min at 4°C. Virus was pelleted at 100,000 g for 45 min and

centrifuged on a sucrose gradient (15–55%) at 100,000 g for 2 h. Purified virus was aliquoted and stored frozen at −70°C. Influenza virus PR/8/34 (H1N1) (a gift from Dr. T. M. Moran, Mount Sinai School of Medicine, New York, NY) was grown in the MDCK cell line (ATCC, Rockville, MD), and the virus was purified from culture supernatants by sucrose gradient centrifugation, as described above. A mouse-adapted virus grown and isolated from the homogenized lungs of intranasally infected mice was used for infection.

ELISA

Influenza-specific antibodies were measured by ELISA. The assay was performed in 96-well polystyrene microtiter plates (Dynatech, Alexandria, VA) coated with purified influenza virus type A/Udorn, at a concentration of 0.5 μg/ml. Dilutions of serum were incubated overnight on coated and blocked ELISA plates, and the bound immunoglobulins were detected with horseradish peroxidase-labeled F(ab′)$_2$ of goat IgG against mouse Ig (Southern Biotechnology Associates, Birmingham, AL). At the end of the incubation time (3 h at 37°C), the peroxidase substrate 2,2′-azino-bis-(3-ethylbenzthiazoline) sulfonic acid (Sigma, St. Louis, MO) in citrate buffer (pH 4.2) containing 0.0075% H_2O_2 was added. The color developed was measured in a Vmax photometer (Molecular Devices, Palo Alto, CA) at 414 nm. The reproducibility of the assay was ascertained by applying on each plate a control hyperimmune mouse serum. The results were expressed as end-point titration values.

Immunizations and challenges of mice

Mice were infected intranasally while awake by depositing 10 μl of virus suspension into the nostrils (5 μl/each nostril). Ketamine-anesthetized mice received 50 μl of virus inoculum for intrapulmonary immunization or TRT infection. For intravenous and intraperitoneal immunizations, 200 μl of virus suspension was injected into the tail vein or the peritoneal cavity of each mouse, respectively. All virus suspensions were adjusted to contain the same amount of virus (10^7 PFU). Before virus challenge, as a proof of successful initial infection, all mice were tested for the presence of virus-specific serum antibody by ELISA. Mice without virus-specific antibodies were excluded from the experiments. For virus challenges, ketamine-anesthetized mice were infected throughout the TRT with 250 PFU (5× LD_{50}) resuspended in 50 μl PBS per animal.

Cells

EL-4 (H-2^b) T cell lymphoma and P815 (H-2^d) mastocytoma cells (ATCC) were maintained in standard complete medium (RPMI 1640; Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS) and antibiotics. Hybridoma 3.155 (TIB-211, ATCC) secretes a rat monoclonal IgM

antibody specific for all mouse Lyt 2 (CD8) alleles. These antibodies can inhibit T-cell-mediated cytolysis in the absence of complement (Sarmiento et al., 1982). Hybridoma GK1.5 (TIB-207, ATCC) produces a rat monoclonal antibody (IgG2b) specific for murine L3T4 (CD4) (Sarmiento et al., 1980).

Generation of antigen-specific CTL effector cells

Effector cells were generated from BALB/c (H-2^d) mice infected by different routes with influenza virus type A, strain Udorn (H3N2), or intravenously with mouse-adapted PR/8/34 (H1N1) (10^7 PFU/mouse). After sacrifice of infected mice, spleens, cervical lymph nodes, lungs, and mediastinal lymph nodes were harvested from five mice per group and single-cell suspensions were pooled for further analysis. A portion of the spleen cell suspension (stimulator cells) was infected with influenza virus type A/Udorn at multiplicity of infection (m.o.i.) of 2–4 or with PR/8/34 at a m.o.i. of 4–6 in a small volume (0.2 ml) of PBS or RPMI 1640 medium without FBS. After incubation for 30 min at 37°C/5% CO_2, RPMI 1640 complete medium was added, and the cell suspension was incubated for at least 3 h, irradiated (3000 R), washed, and mixed with the remaining splenocytes (responder cells) at a ratio of 2:1 (3–4 × 10^6 responder cells/ml) in CTL complete medium containing 10% FBS, 10 mM HEPES, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 0.03% glutamine, and 3 × 10^{-5} M 2-mercaptoethanol. Murine rIL-2 (R&D Systems, Inc., Minneapolis, MN) was added to cultures at day 3 (20 U/ml), followed by an additional 3-day incubation at 37°C/5% CO_2, after which effector cells were washed and tested with virus-infected MHC-matched target cells in a ^{51}Cr release assay.

Preparation of target cells

P815 (H-2^d) and EL-4 (H-2^b) cells were infected at a m.o.i. of 5 with Udorn or PR/8/34 influenza viruses in 100 μl of incomplete medium (without serum) for 20 min at 37°C. The cells were washed to remove unbound virus and were cultured for 2 h in 500 μl of complete medium containing 100 μCi of ^{51}Cr per 10^6 cells. Prior to assessment of cytotoxic activity, ^{51}Cr-labeled cells were washed three times. The cells were counted and used for target cells in the cytotoxic assay, as described below.

Cytotoxic assay

The ^{51}Cr-labeled P815 or EL-4 target cells were washed three times and resuspended in complete medium at 10^5 cells/ml; 100-μl aliquots of the cell suspension were added to 96-well, round-bottom microtiter plates containing triplicate 100-μl samples of serially diluted effector cells. The microtiter plates were centrifuged at 400 g for 5 min and then incubated for 4 h at 37°C/5% CO_2. The level of released radioactivity in 100 μl of supernatant from each well was measured in a

gamma counter (Cobra II Auto-Gamma, Packard Instrument Co., Downers Grove, IL). Specific lysis was calculated from the ^{51}Cr released in counts per minute (cpm) using the formula % specific lysis = (experimental cpm − spontaneous cpm)/(maximal release cpm − spontaneous cpm) × 100; cpm for spontaneous and maximal release was determined by incubating target cells with either 100 μl of medium or 100 μl of 5% Triton X-100, respectively. Spontaneous release of ^{51}Cr in the absence of effector cells was usually less than 15% and did not exceed 20%. SEMs were always less than 5% of the mean value and are not included.

Statistics

Data were analyzed with InStat 2.00. Student's t test (two-tailed) was used for comparison of mean values between groups of mice.

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