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Sendai Virus Pneumonia: Evidence for the Early Recruitment of γδ T Cells during the Disease Course

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We previously reported that γδ T cells appeared and could play a protective role early in infections with intracellular bacteria such as *Listeria monocytogenes*, *Mycobacterium bovis* BCG, and *Salmonella choleraesuis*. To extend these findings to virus infection, we examined the developmental sequence of γδ T cells in bronchoalveolar lavage during the course of Sendai virus infection in C57BL/6 mice. To produce a natural but nonlethal infection course as far as possible, we used a sublethal dose of a wild-type virus which had not been subjected to serial passages in a chicken embryo, hence retaining full virulence for mice. Virus titers in lungs reached a peak on day 6 and then decreased to an undetectable level by day 10. This time course of virus reproduction was immediately and coincidentally followed by the developmental course of γδ T cells, in which the cell number peaked on day 7 and then decreased to a marginal level by day 10. On the other hand, the αβ T-cell number continued to increase until day 10 and remained at a high level thereafter. The early-appearing γδ T cells were CD4$^-$, CD8$^-$, IL-2Rα$^-$β$^+$, CD44$^+$, Mel-14$^-$, and LFA-1α/β$^+$ in phenotype and used Vγ1/2 and Vγ4 and Vδ3, Vδ4, Vδ5, and Vδ6. The γδ T cells were responding to macrophages from infected mice when the cells were cultured in vitro. Furthermore, the expression of endogenous heat shock protein (hsp) was infection specific, and its level appeared to correlate with the γδ T-cell development. These results suggest that the early recruitment of γδ T cells, which proliferate in response to endogeneous hsp$^+$ cells, is also characteristic of this virus infection, although this view appears to be contradictory to earlier reports.

γδ T cells, which represent the first T cells in ontogeny and display more limited diversity than αβ T cells (8, 19, 40), are present only in small numbers in the peripheral lymphoid tissues but are relatively abundant in epithelia of the intestines, skin, and reproductive organs such as the uterus and vagina (30). The ligands of γδ T cells are thought to be allogeneic major histocompatibility complex class I (5, 31), class II (32), and class I-like molecules, such as CD1c (38) and Tla (6). Furthermore, there are several lines of evidence suggesting that a significant fraction of γδ T cells in the newborn thymus (16) and peripheral lymphoid tissues in unprimed adult mice (11) are specified to recognize a murine homolog to the mycobacterial 65-kDa heat shock protein (hsp). hsp-specific γδ T cells were detected in the synovial infiltrates of rheumatoid arthritis patients (22) and the granulomatous lesions of patients with leprosy (34) and tuberculosis (27). γδ T cells increase in infections not only with *Mycobacterium* spp. but with various other pathogens, including *Leishmania donovani* (34), *Plasmodium falciparum malaria* (21), human immunodeficiency virus (4), and Epstein-Barr virus (12). These observations have led to a proposal that γδ T cells may play important roles in protection against infections with diverse pathogens in nature as well as in autoimmune disease processes.

We have previously reported that hsp-specific γδ T cells precede αβ T cells in appearance during infection with *Listeria monocytogenes* (18, 37), *Mycobacterium bovis* BCG (25), and *Salmonella* species (14, 17) in mice, rats, and humans and play a protective role at an early stage in *L. monocytogenes* infection (20). This view was strengthened by the findings of a recent report using γδ gene-targeted mice (35). On the other hand, it has been reported that γδ T-cell receptor (TCR) mRNA$^+$ T cells were prevalent within inflammatory lesions late rather than early in the course of pneumonia caused by influenza A virus (9) or Sendai virus (24) and might therefore be important for the resolution of the inflammatory process. It thus appears that the functional significance of γδ T cells may differ remarkably, depending on the host-parasite system.

The natural host of the Sendai virus is the mouse and several other rodents. The virus has routinely been isolated and propagated by using a chicken embryo as a laboratory host. Serial passages in this host (egg adaptation), however, are known to result in greatly reduced infectivity and pathogenicity for natural hosts. Indeed, in our present system, a 1/1,000 dose of wild-type (WT) virus was sufficient to induce a lethal infection, compared with that of an egg-adapted virus (data not shown). Furthermore, the extent of mononuclear infiltration in a viral respiratory infection was found to correlate with the virulence of the infecting virus (43, 44). Thus, the use of a chicken embryo-adapted Sendai virus (24) might have produced a disease course greatly different from the natural one. This led us to use a WT virus which had not been subjected to serial passages in a chicken embryo in order to reevaluate the role of γδ T cells in the disease course.

Here we show that the appearance of γδ T cells in bronchoalveolar lavage (BAL) is an early event in the acute phase of the host response during Sendai virus infection, which appears to coincide with the expression of endogeneous hsp in alveolar macrophages.

Virus growth and leukocytes in mouse lungs during Sendai

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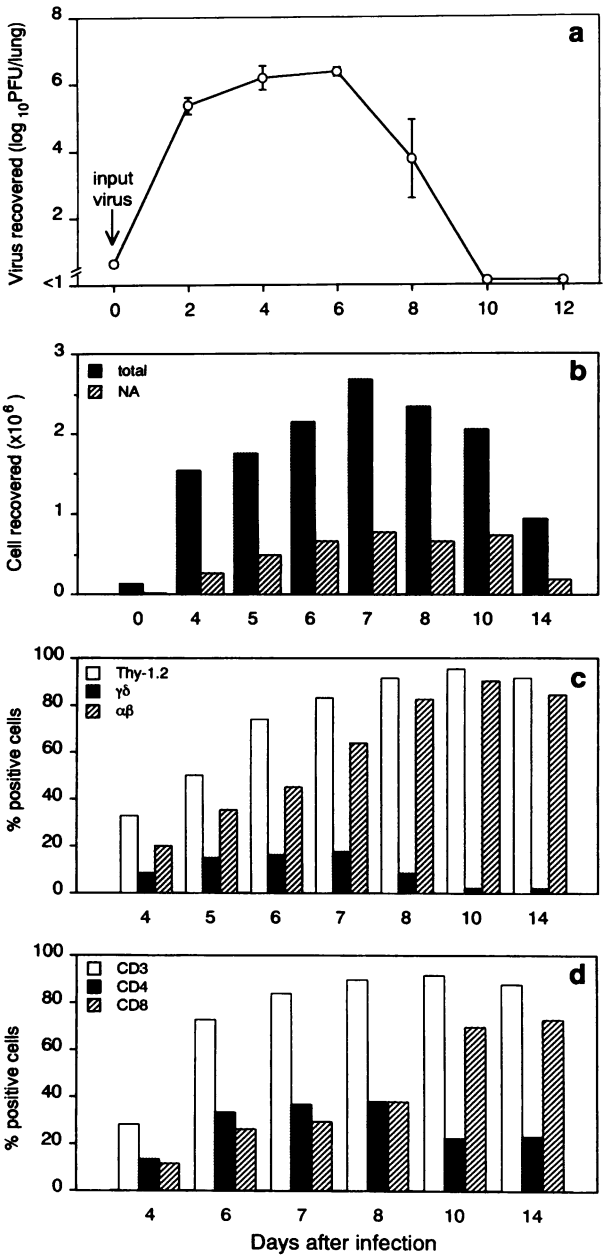

FIG. 1. Virus growth (a) and kinetics of BAL cell development (b through d) in C57BL/6 mouse lungs after an intranasal challenge with WT Sendai virus. Mice were intranasally inoculated with 4.5 PFU of the virus. BAL cells obtained from 2 to 4 groups of infected mice, each consisting of 5 or 6 mice, were used for analyzing the mean number of BAL cells before (total) and after (NA) removal of macrophages by plastic adherence (b); the frequencies of NA BAL lymphocytes expressing Thy-1.2, γδTCR, and αβTCR (c); and the percentages of CD3-, CD4-, and CD8-positive lymphocytes in NA BAL populations (d). NA BAL lymphocytes were stained with phosphatidylethanolamine (PE)–anti-Thy-1.2 MAb, fluorescein isothiocyanate (FITC)–anti-γδTCR MAb, and anti-αβTCR MAb (H57-597) and then reacted with a second antibody, FITC–goat anti-hamster immunoglobulin G (IgG) (c), or they were stained with anti-CD3 MAb (145-2C11), PE–anti-CD4 MAb, and biotin–anti-CD8 MAb and reacted with a second antibody, FITC–goat anti-hamster IgG and Red613-streptavidin (d). The stained cells were analyzed by using a FACScan in the two-color (c) or three-color mode (d).

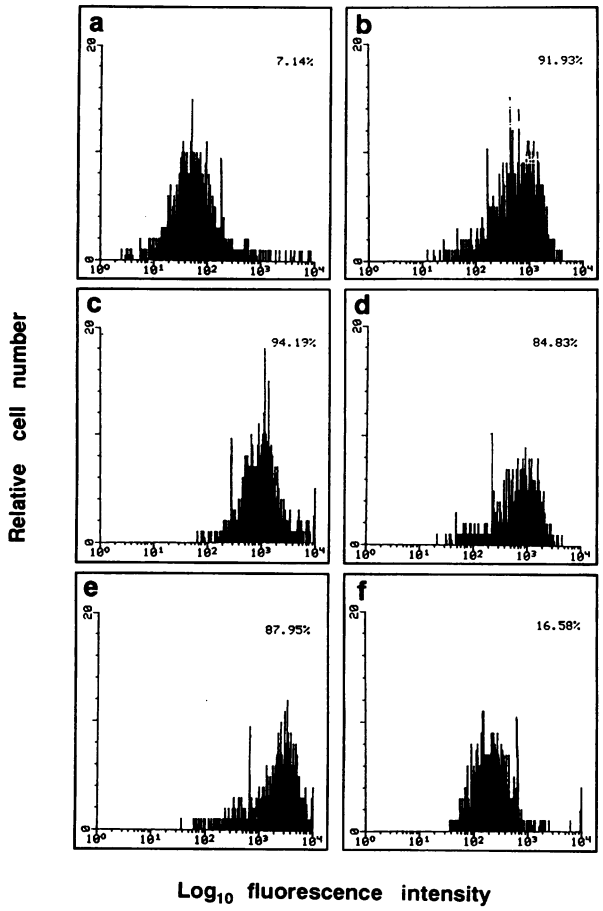

FIG. 2. Surface phenotypic analysis of γδTCR$^+$ T lymphocytes. NA BAL cells that were collected from mice infected 7 days previously were double stained with fluorescein isothiocyanate (FITC)–anti-γδTCR MAb and either biotin–anti-IL-2Rα (a) or anti-IL-2Rβ (b) or with biotin–anti-γδTCR MAb and MAbs to LFA-1α (KBA) (c), LFA-1β (M18/2) (d), Pgp-1 (CD44, IM7; ATCC) (e), or Mel-14 (ATCC) (f). The cells were then stained with PE-streptavidin (a through f) and FITC–goat anti-hamster immunoglobulin G (c through f) and analyzed in the two-color mode. The analysis gate was set on γδTCR$^+$ cells, their expression of each phenotype was represented as a single histogram, and the proportions of stained cells were also represented.

virus infection. The Hamamatsu strain of WT Sendai virus was isolated in an 11-day-old chicken embryo and was from an outbreak of Sendai virus pneumonia in a laboratory mouse colony. The WT virus used in the present study was passaged once more in eggs, plaque purified twice in LLC-MK2 cells, and then propagated in eggs. No further passages were done so that the virus retained full virulence for mice. Pathogen-free inbred 7- to 8-week-old female mice of strain C57BL/6 (Japan SLC) were challenged intranasally under light ether anesthesia with 4.5 PFU of WT virus suspension. After intranasal infection, the virus growth and leukocyte development were examined sequentially. For virus content, 10% of the homogenates of the resected lungs were titrated by plaque assay using the double agar overlay method described previously (2). For leukocyte development, mice were anesthetized with pentobarbitone sodium and subjected to BAL as described previously (28). After centrifugation of lavage fluids, pelleted cells were

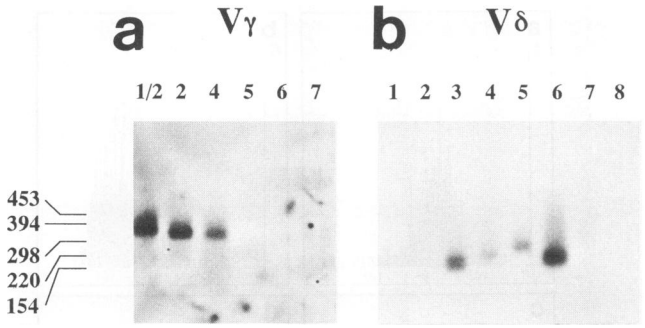

FIG. 3. Vγ (a) and Vδ (b) gene usages of γδ T lymphocytes. Total RNA was extracted from NA BAL cells taken 7 days postinfection, reverse transcribed with a γ chain C region primer or δ chain J region primer, and amplified by PCR with specific V region primers, the nomenclature of which has been described by Itohara et al. (26). Each γ and δ PCR product was subjected to electrophoresis on a 1.8% agarose gel, transferred onto a Hybond N^+ membrane, and probed with ^{32}P-random-labeled MNG6 cDNA containing the Cγ2 gene or Jδ1 probe (5′-TTGGTTCCACAGTCACTTGG-3′) as described previously (14). After hybridization, the membranes were washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature, treated with 0.1× SSC containing 1% sodium dodecyl sulfate at 65°C for 30 min, and autoradiographed. Molecular size is indicated on the left (in base pairs).

resuspended in RPMI medium containing 10% fetal calf serum and incubated in a 12-well plastic plate at 37°C for 90 min to allow macrophages to adhere to the plastic substrate. The resultant nonadherent (NA) cells were processed for two-color or three-color analysis with a FACScan (Becton Dickinson and Co.).

As shown in Fig. 1a, marked virus growth in lungs was detected within 2 days. Thereafter, the virus titer increased, reached a peak on day 6, and then rapidly decreased to an undetectable level by day 10. Severe pneumonia was observed from day 6. Immunohistological examination with a monoclonal antibody (MAb) to the virus fusion glycoprotein on day 6 revealed that virus growth in lungs was restricted to the bronchial epithelium and alveolar macrophages. No virus antigens were found at any place in the lungs on day 10, suggesting complete virus clearance by this day (data not shown).

The total cell number in the BAL continued to increase from $(0.12 \pm 0.02) \times 10^6$ per mouse to $(2.68 \pm 0.30) \times 10^6$ per mouse until day 7 and then gradually decreased (Fig. 1b). The NA subpopulation displayed a very similar time course (Fig. 1b).

The appearance of TCR-bearing lymphocytes was analyzed in parallel for NA BAL populations. Recruitment of γδTCR$^+$ T cells was apparent from the early stages of day 4 (8.3 ± 2.2% of NA BAL lymphocytes) to day 8 (8.5 ± 0.7%), peaking on day 7 (17.6 ± 3.0%) (Fig. 1c). On the other hand, the proportion of αβTCR$^+$ T cells gradually increased over days, peaking on day 10, much later than γδ T cells. The γδTCR$^+$ T cells were already at a marginal level on this day. Thus, αβTCR$^+$ T cells predominated in the late stages of infection (Fig. 1c). Of these αβTCR$^+$ T cells in the late stages, CD8$^+$ cells predominated over CD4$^+$ cells while both CD4$^+$ and CD8$^+$ cells coexisted at the earlier phases before day 8 (Fig. 1d).

Surface phenotypes of γδ T cells. The specific surface phenotypes of γδ T cells were examined by two-color flow cytometric analysis using an anti-γδTCR MAb and phenotype-specific MAbs. Frequent expression of the IL-2Rβ subunit (Fig. 2a) was found on γδTCR$^+$ T cells obtained 7 days postinfection while γδTCR$^+$ T cells hardly expressed the IL-2Rα subunit that is essential for the high-affinity interleukin-2 (IL-2) receptor. The activation marker of lymphocytes, the LFA-1 antigen consisting of α and β subunits (36), was extensively expressed on γδTCR$^+$ T cells (Fig. 2b), the latter subunit being closely related to the cytolytic activity of activated γδ T cells (15). These γδTCR$^+$ T cells were also heavily stained with a MAb to CD44 (Pgp-1), a marker proposed to be specific for the memory T cell, but stained only at a low frequency with a MAb to Mel-14, a marker of the naive T cell (Fig. 2c). This is consistent with the finding of a previous report that both CD44 and LFA-1 are expressed at high levels on acutely activated T cells (23).

V gene segment usage by the γδ T cells. The γδ T cells were found to preferentially use Vγ4 in the early course and Vγ1 in the late course of influenza and Sendai virus pneumonia (9,

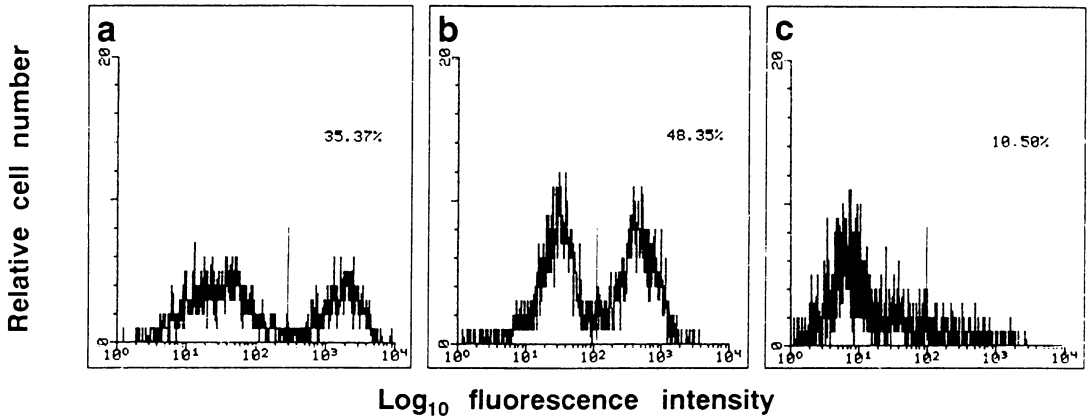

FIG. 4. In vitro proliferation of γδ T lymphocytes. NA BAL cells (5×10^5 per well) recovered from mice infected 7 days previously were immediately stained (a) or stained after coculture for 72 h on a 24-well plate with adherent cells (2×10^5 per well) taken from infected (b) or uninfected mice (c). Staining was done with phosphatidylethanolamine–anti-Thy-1.2 MAb and fluorescein isothiocyanate–anti-γδTCR MAb. The analysis gate was set on Thy-1.2$^+$ cells, their expression of γδTCR was represented as a single histogram, and the proportions of stained cells were also represented.

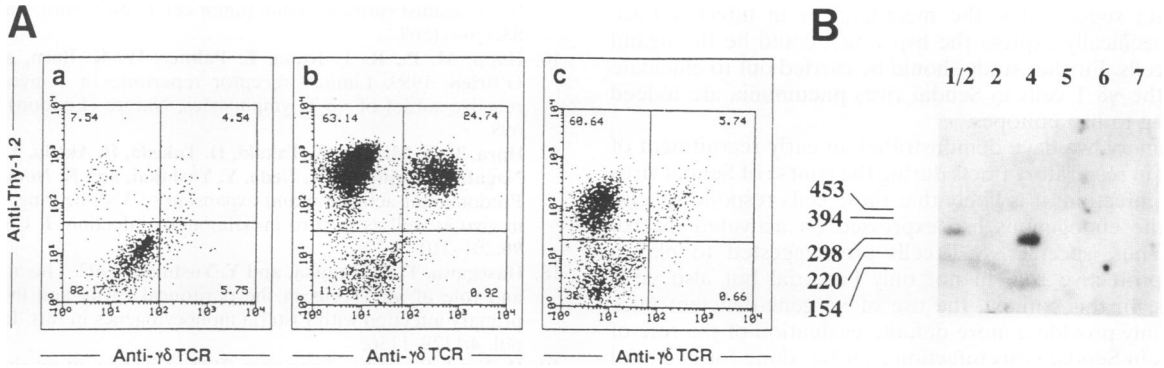

FIG. 5. Analysis of NA BAL cells after priming of C57BL/6 mice with PPDs or OVA. Mice were intranasally administered 15 μl of PPD (1 mg/ml) or OVA (50 mg/ml) for seven consecutive days and then subjected to BAL. (A) NA BAL populations from control (a), PPD-primed (b), and OVA-primed (c) mice were analyzed with a FACScan using phosphatidylethanolamine–anti-Thy-1.2 MAb and fluorescein isothiocyanate–anti-γδTCR MAb. (B) Vγ gene usage of γδ T lymphocytes recovered from PPD-primed mice. Detection of Vγ repertoire was performed as described in the legend to Fig. 3.

24). We determined the Vγ and Vδ gene usages of the early-appearing γδ T cells in the NA BAL fraction during this Sendai virus infection. Total RNA from NA BAL populations 7 days after infection was extracted by the method of Chomczynski and Sacchi (10), reverse transcribed, and amplified by PCR as described previously (14). The PCR products were then analyzed by Southern blotting with specific probes as described previously (14). As shown in Fig. 3, the Vγ and Vδ genes used were predominantly Vγ1/2 and Vδ6. However, Vγ4 was also used but to a lesser extent, and the Vδ repertoire was somewhat diversified.

Expansion of γδ T cells in vitro and induction of γδ T cells by purified protein derivatives. Identification of the ligands recognized by γδ T cells is critical for understanding their function. There is considerable evidence suggesting that the physiological ligand of γδ T cells is an autologous hsp, when they use Vγ1/2 and Vδ6 (11, 16), and that they recognize an epitope in the murine 65-kDa hsp which corresponds to amino acid positions 180 to 196 in the sequence of mycobacterial hsp (7). The same limited repertoire has been shown during infections with BCG (25), *Salmonella choleraesuis* (14), and *L. monocytogenes* (20). Furthermore, pulmonary hsp-reactive γδ T cells in BALB/c mice were reported to predominantly express Vγ4 and Vδ6 (39). The V gene segment usages described above thus suggested that those γδ T cells appearing in Sendai virus infection could also react with the endogenous hsp. We therefore made several attempts to define the ligand(s) of the early-appearing γδ T cells in Sendai virus infection. Firstly, NA BAL cells taken from infected mice were incubated in vitro with syngeneic macrophages from infected mice or with those from normal mice. After 72 h of culture, the percentage of γδ T cells was determined with a FACScan (Fig. 4). It was found that the macrophages from infected mice continuously stimulated the γδ T cells and allowed them to proliferate, whereas those from normal mice did not induce such an expansion of the γδ T cells at all (Fig. 4b and c). Secondly, BAL cells from purified protein derivative (PPD)- and ovalbumin (OVA)-primed mice were compared. In the PPD-primed mice, the proportion of γδ T cells in the respiratory lining fluid was obviously increased (Fig. 5A, panel b), whereas little increase was found for the OVA-primed mice (Fig. 5A, panel c). These results are consistent with the previous finding that the expansion of γδ T cells was prominent when pulmonary lymphocytes were primed with PPDs either in vivo or in vitro (3). The γδ T cells proliferating in response to PPDs were found to preferentially use Vγ4 and Vγ1/2 (Fig. 5B) and are similar in Vγ gene usage to Sendai virus-infected mice. Therefore, a component(s) shared by PPD and activated alveolar macrophages, i.e., hsp, appeared to be the ligand of these proliferating γδ T cells.

Finally, we carried out an immunoblot analysis with the anti-mycobacterial 65-kDa hsp MAb IA10 in macrophages sequentially collected from infected mice to learn whether these cells express the endogenous hsp. This MAb, IA10, was raised against an epitope in mycobacterial hsp which is conserved in both prokaryotic and eukaryotic hsps and was shown to react with murine hsp (29). As shown in Fig. 6, several protein bands with apparent molecular masses of around 60 kDa (33, 42) were detected by the antibody throughout infection from day 4. These bands were not detected in control macrophages and were therefore infection specific, and the elevation in expression of these bands correlated with the development of γδ T cells, at least in the early stages. Later, however, hsp remained at a high level while the γδ T-cell number decreased. It is likely that the primed αβ T cells proliferating at this late phase are secreting lymphokines and cytokines that facilitate hsp65 transcription (1, 13, 24, 29, 41).

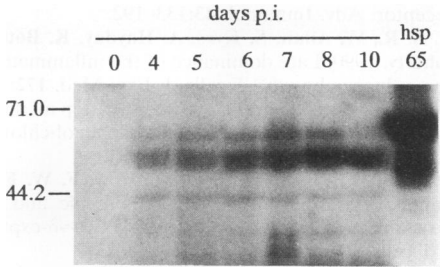

FIG. 6. Identification of 65-kDa hsp cross-reactive proteins in BAL macrophages from Sendai virus-infected mice. Lysates of BAL macrophages (1×10^5 per lane) taken from infected mice on various days postinfection (p.i.) or uninfected mice (day 0) were electrophoresed under the reducing conditions. The separated proteins were blotted onto a polyvinyl membrane and probed with MAb IA10 and, after being washed, reacted with the second antibody, rabbit anti-mouse immunoglobulin G. To visualize specific bands, the membrane was reacted with ^{125}I-protein A and autoradiographed. Mycobacterial 65-kDa hsp (hsp65) was run as a positive marker. Molecular size is indicated on the left (in kilodaltons).

These data suggest that the macrophages in infected mice indeed specifically express the hsp which could be the ligand for γδ T cells. Further study should be carried out to elucidate whether the γδ T cells in Sendai virus pneumonia are indeed responding to hsp epitopes.

In summary, we have demonstrated an early recruitment of γδ T cells in respiratory tracts during the course of Sendai virus sublethal infection; it is likely that these cells respond specifically to the endogenous hsp expressed on activated macrophages. Thus, specific γδ T cells are suggested to play a primary protective role in not only bacterial but also virus infections. In this context, the use of γδ gene-deficient mice will certainly provide a more definite evaluation of the role of γδ T cells in Sendai virus infection as it has done for bacterial infection (35).

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