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Selective Response of γδ T-Cell Hybridomas to Orthomyxovirus-Infected Cells

SATHIBALAN PONNIAH,* PETER C. DOHERTY, AND MARYNA EICHELBERGER

Department of Immunology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105

Received 7 April 1995/Accepted 21 September 1995

A γδ T-cell hybridoma established from influenza virus-infected mice responded in a reproducible way when cultured with influenza virus-infected stimulators. Subclones of this line responded to cells infected with influenza viruses A/PR/8/34 (H1N1), X-31 (H3N2), and B/HK/8/73 but not to cells infected with vaccinia virus or Sendai virus. This spectrum of response to both type A and type B orthomyxoviruses has never been recognized for the αβ T-cell receptor-positive subsets. There was no response to cells infected with a panel of recombinant vaccinia viruses expressing all individual influenza virus proteins, and so it is unlikely that the stimulating antigen is of viral origin. The alternative is that the antigen is a cellular molecule induced in influenza virus-infected cells. Infectious virus was required for stimulation, and immunofluorescence studies showed increased expression of heat shock protein 60 (Hsp60) in influenza virus- but not Sendai virus- or vaccinia virus-infected cells. Both the hybridoma generated from influenza virus-infected mice and an established hybridoma which uses the same γδ T-cell receptor combination responded to recombinant Hsp60. Furthermore, the Hsp60-reactive hybridoma, which was obtained from an uninfected mouse, also responded to influenza virus-infected cells, indicating that Hsp60 may indeed be the target antigen.

The role of γδ T cells in immunity is not yet clear. They contribute early in the response to infection with *Listeria monocytogenes* (16) and have been isolated from sites of inflammatory pathology in mice infected with various bacteria, parasites, and viruses (9). Evidence of γδ T-cell specificity for a pathogen has, however, been demonstrated only for herpes simplex virus type 1; in this case, the glycoprotein gI is recognized by the γδ T-cell receptor (TCR) as a soluble molecule and not in association with major histocompatibility complex (MHC) glycoproteins (13, 21).

The possibility that γδ T cells are involved in the response to influenza virus is suggested by the fact that activated lymphocytes of this subset are present in the site of inflammatory pathology (2, 5). These γδ T cells could potentially be responding to infected cells expressing viral protein or to cells expressing a modified (or overexpressed) cellular molecule. Late after infection (days 10 and 13 postinfection), heat shock protein 60 (Hsp60) mRNA is upregulated in phagocytic cells isolated by bronchoalveolar lavage of mice infected intranasally with influenza virus (1, 4). At a similar time period, $V\gamma1^+$ T cells, which are known to respond to bacterial Hsp60 (10, 18), infiltrate the pneumonic lung. It has thus been suggested that these inflammatory γδ T cells may be responding to this induced Hsp60.

The present analysis concentrates on the response of γδ TCR^+ hybridomas generated from mice homozygous for a disruption in the TCRβ gene locus ($TCR\beta^{-/-}$ mice) (17) that were infected with an influenza A virus.

MATERIALS AND METHODS

Mice. $TCR\beta^{-/-}$ mice were established on the 129/Ola (*H-2*^{b}) × BALB/c (*H-2*^{d}) background at the Massachusetts Institute of Technology laboratories (17). They were maintained under specific-pathogen-free conditions at the Animal Resource Center of St. Jude Children's Research Hospital until infected with influenza virus.

Viruses. Influenza viruses X-31 (H3N2), A/PR/8/34 (PR8; H1N1), and B/HK/8/73 (B/HK; HG) and Sendai virus were grown in the allantois of embryonated chicken eggs (3), and aliquots were stored at −70°C until use. Wild-type vaccinia virus (strain WR) and recombinants (23) were generously provided by Jack Bennick.

Cell lines. The macrophage cell line LIE13.14 (MHC class I^+ II^+) was established by culture of cells lavaged from the lungs of influenza virus-infected C57BL/6 (*H-2*^{b}) mice in the presence of colony-stimulating factor 1 (CSF)-1 and subsequent cloning at limiting dilution (26). These cells, as well as $P388D_1$ cells (*H-2*^{d}), were maintained as adherent cells on polymethylpentene petri dishes (Nalgene) (20) in RPMI (Life Technologies Inc.) supplemented with 10% fetal calf serum (FCS) and 20% supernatant of Ladmac cells. The CSF-1-producing Ladmac cells (22) were cultured for 6 to 7 days in Eagle minimal essential medium (Life Technologies Inc.) containing 10% FCS before the supernatant was removed and aliquots were frozen. MHC class I^+ II^- cell lines P815 (*H-2*^{d}), MC57G (*H-2*^{b}), and L929 (*H-2*^{k}) were cultured in RPMI containing 10% FCS. The macrophage line AM11 was derived from a bulk culture of LIE13 cells which were transformed with J2 retrovirus (20) to facilitate growth in the absence of CSF-1.

Purified proteins. Recombinant human Hsp60 and Hsp70 were purchased from StressGen Biotechnologies Corp. (Victoria, British Columbia, Canada).

Hybridomas. $TCR\beta^{-/-}$ mice were infected with influenza virus X-31 (H3N2) as described previously (2). Cells from the draining mediastinal lymph nodes were cultured in S minimal essential medium (Life Technologies Inc.) containing 10% FCS, supplements (15), and concanavalin A (2 μg/ml). After 3 days in culture, the cells were fused with BWα-β-thymoma cells (25) and hybridomas were selected at limiting dilution in the presence of hypoxanthine-aminopterin-thymidine. The hybridomas were subcloned at limiting dilution and cultured in supplemented S minimal essential medium. γδ T-cell hybridoma BNT19.8.12 was kindly provided by W. Born, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colo. αβ T-cell hybridomas 11.2.2, 12.64, and 12.91 are all specific for a peptide from the nucleoprotein of influenza A virus strains (3) and were used as controls.

Immunofluorescence. Lymphocytes were phenotyped by surface immunofluorescence, using purified, fluorescein isothiocyanate (FITC)- or phycoerythrin-conjugated anti-αβ TCR (H57-597) and anti-γδ TCR (GL3) monoclonal antibodies (MAbs) purchased from PharMingen (San Diego, Calif.). The analysis was done with a FACScan (Becton Dickinson, Mountain View, Calif.) as described previously (5).

Fluorescence microscopic analysis of Hsp expression was done with LIE13.14 cells grown in eight-well multichamber slides (Nunc, Naperville, Ill.). When a confluent layer was achieved, the medium was removed and cells were infected with 10 infectious virus particles per cell for 1 h. Fresh medium was then added to the wells, which were returned to culture for a further 8 to 12 h. Staining with a MAb specific for influenza A virus and Sendai virus nucleoprotein (NP) molecules showed good infection of all cells. The cells were then fixed by immersing the slides for 2 min in a 1:1 mixture of methanol-acetone which had

* Corresponding author. Mailing address: Department of Immunology, St. Jude Children's Research Hospital, 332 N. Lauderdale, Memphis, TN 38105. Phone: (901) 495-3470. Fax: (901) 495-3107. Electronic mail address: sathibalanponniah@mbcf.stjude.org.

TABLE 1. Specificity of γδ T-cell hybridomas

Clone	Avg cpm of [^{3}H]thymidine incorporated into triplicate cultures of CTLL cells ± SD in supernatant from hybridoma cultured with:					
	Medium	Y-CD3	AM11 (*H-2^b*)		P338D$_1$ (*H-2^d*)	
			Uninfected	X-31 infected	Uninfected	X-31 infected
2.1	786 ± 478	24,724 ± 2,024	2,283 ± 2,667	1,094 ± 170	580 ± 68	2,246 ± 11
2.3	3,188 ± 269	13,448 ± 529	11,797 ± 697	15,046 ± 948	4,895 ± 126	15,903 ± 1,052
2.8	618 ± 135	24,313 ± 406	528 ± 88	1,049 ± 219	431 ± 73	679 ± 117
2.11	461 ± 51	8,727 ± 298	509 ± 62	845 ± 257	452 ± 8	1,303 ± 362
2.15	750 ± 129	8,630 ± 104	659 ± 92	569 ± 25	681 ± 193	708 ± 79
2.17	1,026 ± 144	27,567 ± 932	1,077 ± 169	1,762 ± 1,766	1,489 ± 982	984 ± 83

been chilled at −20°C. The slides were air dried or left overnight at −70°C, after which they were incubated with antibodies specific for Hsp or viral components. The Hsp-specific MAbs (StressGen) included mouse anti-Hsp60 (clone LK2), mouse anti-Hsp72 (C92F3A-5), and rat anti-Hsc70 (clone 1B5). Mouse anti-influenza virus NP was used as a supernatant from clone 46-4 (American Type Culture Collection). Mouse anti-Sendai virus NP was a gift from A. Portner. The slides were then washed in phosphate-buffered saline containing 2% bovine serum albumin and stained with FITC-conjugated reagents. FITC-conjugated goat anti-mouse immunoglobulin was obtained from Becton Dickinson. FITC-conjugated goat anti-rat F(ab′)$_2$, as well as control mouse and rat immunoglobulins, were purchased from Southern Biotechnology (Birmingham, Ala.). After washing, the cells were viewed under a Nikon Biophot fluorescence microscope and photographed with a Nikon camera.

Stimulation assay. Antigen-presenting cells were infected with virus (10 egg infectious influenza virus particles per cell or 10 PFU of vaccinia virus per cell) for 4 h before culture with hybridomas. Hybridomas were cultured at 1×10^4 or 2×10^4 per well in 200 μl of complete medium in the presence or absence of equal numbers of antigen-presenting cells. As a positive control, hybridomas were incubated with irradiated Y-CD3 hybridoma cells (19) as a source of cross-linked anti-CD3ε. Cultures were incubated for 18 to 24 h at 37°C in 5% CO_2. Supernatants were harvested and tested for the presence of interleukin-2 (IL-2), using the CTLL indicator cell line (8). The CTLL cells were cultured with triplicate or quadruplicate samples of supernatants, and proliferation was measured by incorporation of [^{3}H]thymidine during the last 6 h of a 24-h culture period.

When protein was used as the antigen, wells of a flat-bottom microtiter plate were coated with 4 to 0.125 μg per well for 3 h at 37°C. The wells were washed three times with S minimal essential medium before addition of the hybridoma cells. Antibody was added to wells coated with Hsp60 at 4 μg per well, or to wells containing infected cells at 10 μg per well, 3 h before addition of the hybridoma cells.

RESULTS

Establishment of γδ T-cell hybridomas. Hybridoma cell lines were generated by fusing concanavalin A-stimulated mediastinal lymph node cells pooled from five TCRβ$^{-/-}$ mice that had been infected intranasally with the X-31 virus 28 days previously. The responder characteristics of a representative panel of *H-2^{d+}-H-2^{b-}* TCRδ$^+$β$^-$ hybridomas (it is likely that the *H-2^b* allele is lost) are shown in Table 1. Enhanced IL-2 production was observed for all cell lines following cross-linking with immobilized anti-CD3ε (Y-CD3; Table 1). Clones 2.1 and 2.3 secreted more IL-2 following culture with the X-31-infected macrophage P338D$_1$ (*H-2^d*) cell line (Table 1). However, the response pattern of clone 2.1 was inconsistent in repeat experiments (data not shown) and was not pursued further. In addition, clone 2.3 responded to influenza virus-infected AM11 cells (*H-2^b*), though substantial amounts of IL-2 were also produced when uninfected AM11 cells were used as stimulators.

Hybridoma 2.3 was thus subcloned by limiting dilution, and much of the further analysis was restricted to subclones which expressed significant levels of the γδ TCR, as detected by fluorescence-activated cell sorting analysis for the cell surface expression of TCRδ (data not shown). The presence of TCRδ and the absence of TCRβ were further confirmed by measuring levels of IL-2 production following culture with an immobilized antibody (Fig. 1). Clearly the T cells were induced by exposure to MAb GL3, specific for TCRδ, but not by MAb H57-597, specific for TCRβ, while the reverse was true for the αβ hybridoma 12.91. The TCRs on the subclones of clone 2.3 were found by PCR to express variable regions Vγ1 and Vδ6.

Specificity for virus. The next question was whether the non-MHC-restricted response to the influenza A virus X-31 (H3N2)-infected stimulators, inferred from the initial results in Table 1, was indeed virus specific. The LIE13.14 cells were thus infected with X-31 or influenza virus B/HK. Cross-reactivity for the influenza A and B viruses has not been recognized for αβ T cells. The controls were uninfected cells and cells treated with normal allantoic fluid. Several of the subclones responded consistently to cells infected with either the influenza A or the influenza B virus (Fig. 2, clones 2.3.4 and 2.3.5), while other subclones (2.3.2, 2.3.8, and 2.3.10) varied in their responses between experiments, although these cells always responded in the presence of anti-γδ TCR.

The hybridomas were then cultured with LIE13.14 cells that were either infected with influenza A virus PR8 (H1N1) or treated with heat-inactivated PR8. Specific IL-2 secretion was observed only when infectious virus was present, as demon-

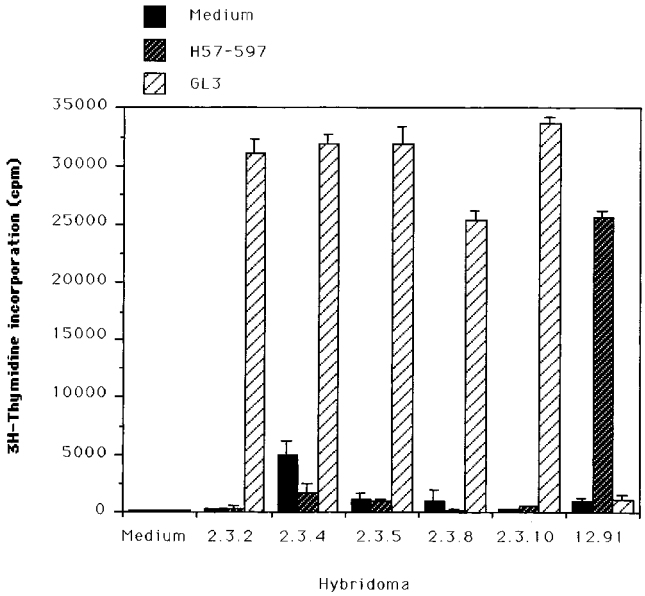


FIG. 1. IL-2 released by hybridoma subclones when cultured with antibodies specific for αβ or γδ TCR. Hybridoma cells were cultured on their own, with anti-αβ TCR MAb H57.597, or with anti-γδ TCR MAb GL3-3A. Results are shown as the average [^{3}H]thymidine incorporated (counts per minute) into CTLL cells cultured in the presence of the hybridoma cell supernatants. The standard deviation is shown on each bar.

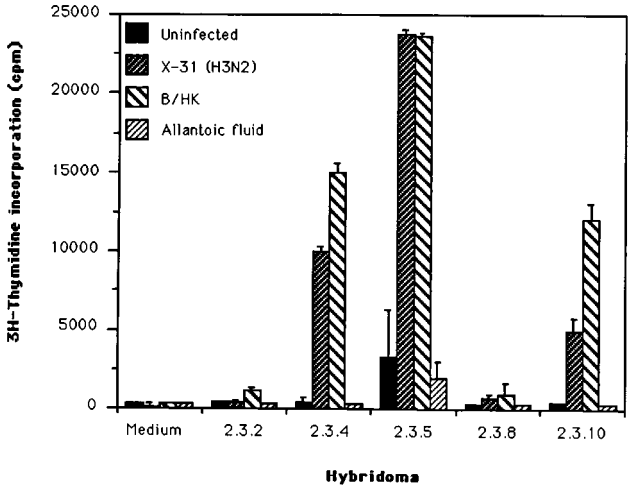


FIG. 2. Stimulation of hybridoma cells with influenza virus-infected LIE13.14 cells. LIE13.14 cells were uninfected, infected with X-31 (H3N2) or B/HK (HG), or treated with allantoic fluid from uninfected eggs. Results are shown as the average [^{3}H]thymidine incorporated (counts per minute) into CTLL cells cultured in the presence of the hybridoma cell supernatants. The standard deviation is shown on each bar.

strated by the response of the influenza A NP-specific αβ TCR hybridoma 12.91 to cells treated with live virus and not the inactivated virus (Fig. 3). This result suggests that infection is necessary and/or that killed virus cannot be processed via a class II MHC-dependent pathway to give an appropriate antigenic complex.

We then examined whether the cross-reactive stimulation seen for the influenza A and B viruses (Fig. 2 and 3) extends beyond the orthomyxoviruses. The LIE13.14 cells were thus infected with another RNA virus, a murine paramyxovirus (Sendai virus), or with a DNA virus (vaccinia virus), using protocols that are in regular use in the laboratory to stimulate αβ T cells specific for these two viruses (references 11 and 24 and data not shown). The dose of virus used to infect the

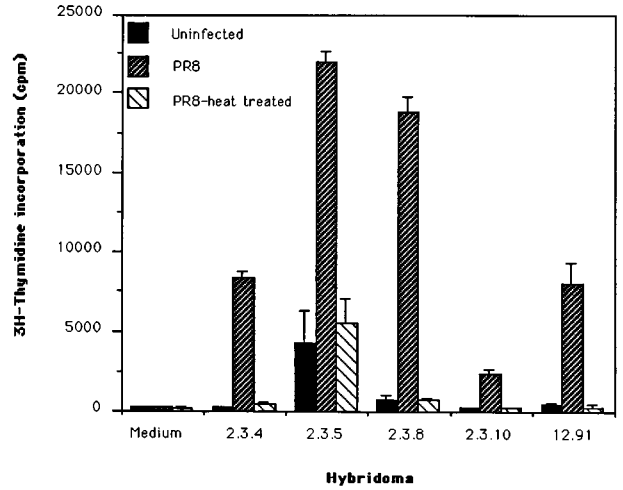
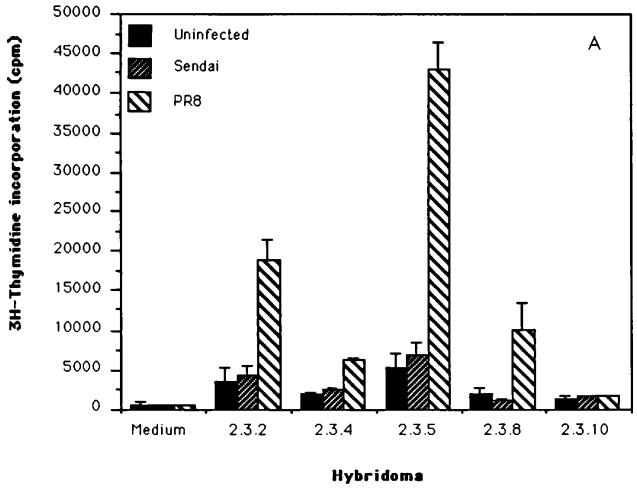


FIG. 3. Responses of hybridoma cells stimulated with LIE13.14 cells alone, cells infected with PR8 (H1N1), or cells in the presence of the same virus which was rendered noninfectious by heat treatment. Results are shown as the average [^{3}H]thymidine incorporated (counts per minute) into CTLL cells cultured in the presence of the hybridoma cell supernatants. The standard deviation is shown on each bar.



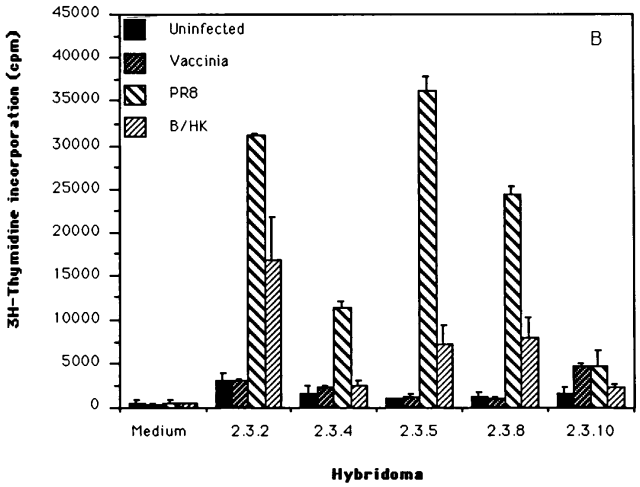


FIG. 4. (A) Responses of γδ hybridomas to uninfected or Sendai virus- or influenza A virus PR8-infected LIE13.14 cells. (B) Responses of γδ hybridomas to uninfected or vaccinia virus (strain WR)-, influenza A virus PR8-, or influenza virus B/HK-infected LIE13.14 cells.

LIE13.14 cells was 10 particles per cell in all cases, and there was no difference in the viability of cells infected with different viruses at the initiation of the stimulation assays. Only the influenza virus-infected cells induced substantial IL-2 production by the γδ hybridomas (Fig. 4). The results thus indicate that there is specificity for the orthomyxoviruses.

Lack of specificity for an individual influenza virus protein. The hybridomas were incubated with LIE13.14 cells which had been infected with vaccinia virus recombinants expressing a complete panel of influenza virus proteins. Although the expected reactivity was obtained for the αβ T-cell hybridoma response to PR8 and NP, no single influenza virus protein was able to stimulate the γδ T-cell hybridoma to respond (Table 2).

Correlation with Hsp expression. Since we obtained different responses to cells infected with different viruses, the obvious question was whether this variation might be correlated with the differential expression of endogenous Hsp. The LIE13.14 cells were thus infected with influenza A virus PR8, Sendai virus, or vaccinia virus, stained with MAbs to viral proteins or to various Hsps, and analyzed by fluorescence mi-

TABLE 2. Responses of T-cell hybridomas to influenza virus proteins expressed by vaccinia virus recombinants

Infecting virus[a]	cpm[b] in responding hybridoma:		
	2.3.5	2.3.8	12.64[c]
None (medium)	1,650	1,124	1,327
PR8	13,510	14,954	36,791
B/HK	25,294	21,802	1,155
Vacc-hemagglutinin H1	2,172	849	1,207
Vacc-hemagglutinin H2	2,422	637	1,145
Vacc-hemagglutinin H3	4,617	537	1,210
Vacc-neuraminidase N1	2,548	712	1,027
Vacc-NP	1,830	701	40,093
Vacc-polymerase PB2	3,294	761	1,517
Vacc-polymerase PB1	2,161	764	1,061
Vacc-polymerase PA	2,324	644	1,125
Vacc-nonstructural protein NS1	1,804	608	1,230
Vacc-nonstructural protein NS2	2,468	554	788
Vacc-matrix protein M1	1,642	556	839
Vacc-matrix protein M2	2,878	513	1,167

[a] The vaccinia virus (Vacc) recombinants were constructed and provided by Jack Bennick. The origin of each protein was as follows: N1, A/CAM/46; H2, A/Jap/57; H3, A/NT/60/68; NS2, A/Udorn/72; all other proteins, A/PR/8/34. Virus stocks were made by Kieran Daly. LIE13.14 cells were infected by using a standard protocol.

[b] Average of triplicate CTLL cultures. The standard deviation was less than 10% of the counts.

[c] A D^b-restricted αβ T-cell hybridoma specific for NP (3).

croscopy (Fig. 5). Antibodies to virus-specific proteins were used to verify successful infection of the LIE13.14 cells (Fig. 5F and K). Infection of the cells with influenza A virus PR8 resulted in increased expression of Hsp60 (compare Fig. 5N with Fig. 5M) and, to a lesser extent, Hsc73 (compare Fig. 5V with Fig. 5U). There was also induced expression of Hsp72 in some cells (compare Fig. 5R with Fig. 5Q). In contrast, cells which were infected with either vaccinia virus or Sendai virus did not show similar increases. Indeed, decreased expression of Hsps has previously been found in cells infected with vaccinia virus (12).

Response to Hsp60. Murine γδ T cells which utilize Vγ1Vδ6 TCRs have often been characterized as Hsp60 reactive. Indeed, hybridomas 2.3.5 and 2.3.8 do respond to recombinant human Hsp60 but not to recombinant Hsp70 (which have approximately 80% homology with murine Hsp60 and Hsp70, respectively) (Table 3). Hybridoma BNT19.8.12, previously characterized as Hsp60 reactive (6, 7), also responded to orthomyxovirus-infected cells (Table 4).

DISCUSSION

We have demonstrated that some γδ T cells have apparent specificity for orthomyxovirus-infected cells. This response is not MHC restricted, since the hybridomas could respond to both *H-2*^{b}(LIE13.14) and *H-2*^{d}($P388D_1$) macrophage lines. In addition, class I^+ II^- cell lines MC57G (*H-2*^{b}), P815 (*H-2*^{d}),

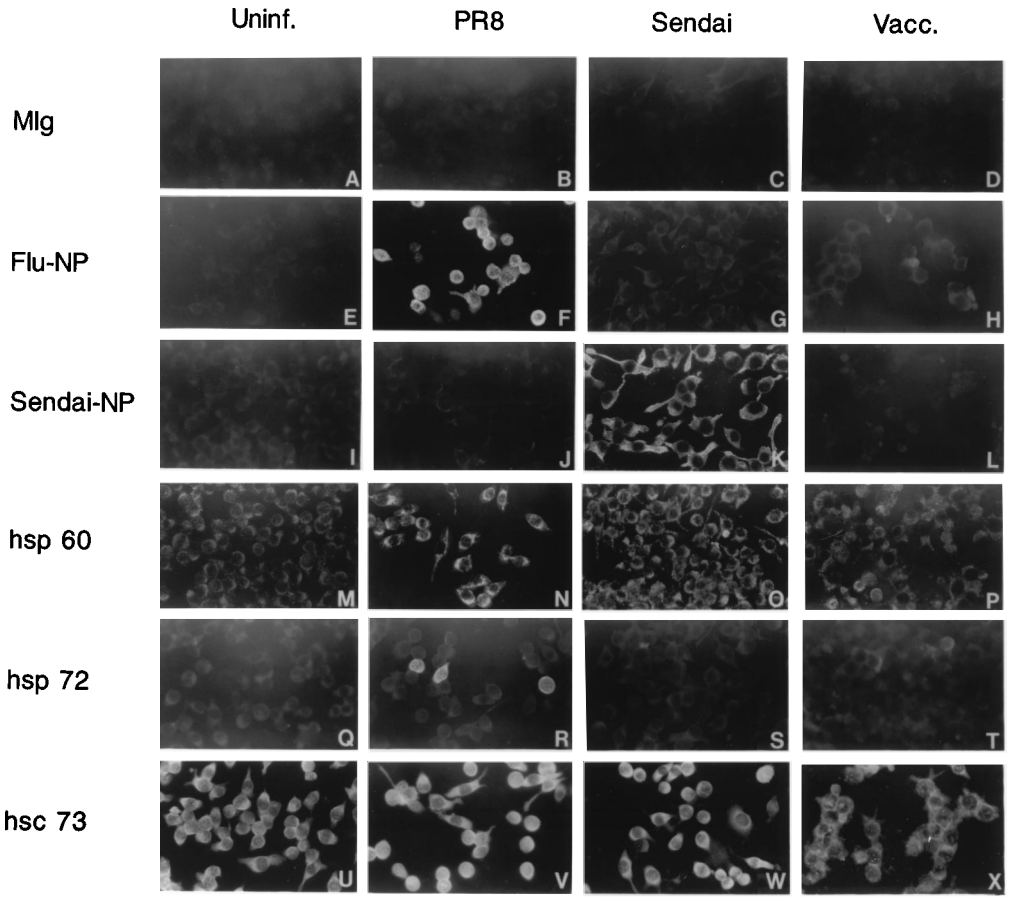


FIG. 5. Immunofluorescence staining of LIE13.14 cells uninfected (Uninf.) or infected with influenza A virus PR8, Sendai virus, and vaccinia virus (strain WR) (Vacc.). LIE13.14 cells were infected for 10 h, fixed with ethanol, and then stained by using antibodies specific for mouse immunoglobulin (MIg) as a negative control, influenza virus NP (Flu-NP), Sendai virus NP (Sendai-NP), Hsp60, Hsp72, and Hsc73.

TABLE 3. Responses of γδ T-cell hybridomas to recombinant heat shock proteins[a]

Expt	Hybridoma	Avg cpm of [^{3}H]thymidine incorporated into CTLL cells ± SD		
		No protein	Hsp60	Hsp70
1	2.3.5	458 ± 24	45,975 ± 2,428	1,123 ± 745
	2.3.8	521 ± 105	27,860 ± 1,752	526 ± 147
2	2.3.5	1,638 ± 122	35,847 ± 1,282	ND
	2.3.8	1,388 ± 211	16,815 ± 653	ND
	12.64[b]	1,327 ± 153	1,251 ± 645	ND

[a] Both recombinant human Hsp60 and recombinant human Hsp70 were purchased from StressGen. They were added to hybridoma cells at 1 μg/ml. The stimulation assay described in Materials and Methods was used. ND, not done.
[b] An αβ T-cell hybridoma with specificity for the influenza virus NP epitope (3).

and L929(*H-2^k*) could be used to stimulate these γδ T-cell hybridomas (data not shown).

Throughout this study, responses by the same hybridoma cell line varied considerably. The degree to which the hybridoma was stimulated may reflect varying levels of Hsp expression through the cell cycle (14) or stages of growth in vitro of both the hybridoma and the stimulator cells. It is possible that the variability may be due to the numbers of TCR heterodimers and/or adhesion molecules on the surface of the hybridomas. The cell lines were analyzed by flow cytometry for expression of cell surface molecules but showed no pattern that correlated with the level of biological activity being detected. All hybridomas expressed approximately equivalent levels of TCRδ, CD11a, CD44, and CD49d but did not express much, if any, CD2, CD28, CD54, and CD62L (data not shown). However, it is possible that other adhesion molecules that were not analyzed may be differentially expressed at the surface of these cells. In addition, there may also be variation in the expression levels of the putative Hsp60 antigen by the infected cells in the different experiments. However, hybridomas 2.3.5 and 2.3.8 seemed to eventually be the most consistent and stable of the panel studied and thus were chosen to define the responses to the purified Hsp60 protein.

The γδ hybridoma panel responds to both influenza A and influenza B virus-infected cells but not to Sendai virus- or vaccinia virus-infected cells. This pattern of selective response toward orthomyxovirus-infected cells has never been seen for conventional CD4$^+$ or CD8$^+$ αβ T cells. The proteins from these viruses have little homology (10 to 37%), except for polymerase protein PB1, which has 61% homology. It seemed possible that the TCRs of these hybridomas were interacting with a conserved region from one of these viral proteins or perhaps were directed toward a conformational epitope which need not have total sequence homology. Lack of reactivity with a complete panel of influenza virus proteins expressed in stimulator cells suggests that this is not the case.

TABLE 4. An Hsp60-reactive γδ T-cell hybridoma responds to influenza virus-infected cells

Hybridoma[a]	Avg cpm of triplicate CTLL cultures of LIE13.14 cells treated with[b]:	
	Allantoic fluid	PR8
12.64	793	41,890
BNT19.8.12	6,194	77,621
2.3.5	1,144	17,452

[a] Hybridoma 12.64 is an αβ T-cell hybridoma with specificity for a peptide of NP of influenza A virus (3); 2.3.5 is a γδ T-cell hybridoma; BNT19.8.12 is a γδ T-cell hybridoma with apparent specificity for Hsp60 (6, 7).
[b] LIE 13.14 cells were infected with influenza virus or allantoic fluid, and the cells were washed before plating at 2×10^4 cells per well. Supernatants were harvested 12 h after addition of the hybridoma cells and assayed for the presence of IL-2, using the standard CTLL cell line. The standard deviation was less than 10% of the counts.

The antigen which is recognized is therefore likely to be a cellular molecule which is induced (or upregulated) during the infection with influenza virus. Since there was no stimulation with either Sendai virus or vaccinia virus, such upregulation should be specific for orthomyxoviruses. Immunofluorescence staining for Hsp molecules indicated that Hsp60 expression was increased only in the cells infected with the influenza A or influenza B virus, suggesting that Hsp60 may be the antigen recognized. Although some PR8-infected cells had increased expression of Hsp72 as detected by immunofluorescence, the hybridoma cells did not respond to recombinant Hsp72. The response to Hsp60 was not due to contamination of this preparation with lipopolysaccharide (LPS) from the bacteria in which the recombinant protein was expressed, since LPS does not stimulate these γδ T-cell hybridomas (results not shown). This response, together with the increased expression of Hsp60 during infection with influenza virus, suggests that Hsp60 is indeed the antigen recognized by these γδ T-cell hybridomas. In addition, BNT19.8.12, a γδ TCR hybridoma which originated from the thymus of uninfected mice and is characterized as Hsp60 reactive (6, 7), responded to the orthomyxovirus-infected cells. At the moment, we have no conclusive evidence for the direct involvement of the γδ TCR in the recognition process for the observed responses in our experiments. However, variants of hybridoma BNT19.8.12 and our hybridomas which have lost expression of the γδ TCR at their surface were observed to be nonresponsive to the influenza virus-infected cells, thereby strongly supporting the involvement of the γδ TCR in the responses observed. Our laboratory is currently using biochemical techniques to define the antigen on the surface of influenza virus-infected cells which is recognized by these γδ T-cell hybridomas.

Since it is clear that there is reactivity toward orthomyxovirus-infected cells but that exposure to virus in vivo is not required for the establishment of this response, there is the potential for γδ T cells to respond to influenza virus infection. If this reactivity is indeed directed toward the Hsp60 molecule, it can be envisioned that γδ T cells may play important roles at different times after infection. Early in the infection, γδ T cells may directly interact with virus-infected cells or regulate the immune response in an antigen-specific manner. Once influenza virus is cleared (10 to 13 days after infection), there is upregulation of Hsp60 mRNA levels in inflammatory macrophages. Much of this effect seems to be induced by cytokines produced by the responding CD4 and CD8 T cells (1). At the same time, the Vγ1$^+$ T cells are at highest concentration (2). It is possible that Vγ1$^+$ T cells respond to the elevated level of Hsp60 expression and consequently secrete lymphokines (5) to initiate a phase of recovery in the lung or, alternatively, to maintain macrophage activation and protect from secondary bacterial infection.

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REFERENCES

1. **Allan, W., S. R. Carding, M. Eichelberger, and P. C. Doherty.** 1993. hsp65 mRNA$^+$ macrophages and γδ T cells in influenza virus-infected mice depleted of the CD4$^+$ lymphocyte subsets. Microb. Pathog. **14:**75–84.
2. **Carding, S. R., W. Allan, S. Kyes, A. Hayday, K. Bottomly, and P. C. Doherty.** 1990. Late dominance of the inflammatory process in murine influenza by γ/δ$^+$ T cells. J. Exp. Med. **172:**1225–1231.
3. **Deckhut, A. M., W. Allan, A. McMickle, M. Eichelberger, M. A. Blackman, P. C. Doherty, and D. L. Woodland.** 1993. Prominant usage of Vβ8.3 T cells in the H-2D^b-restricted response to an influenza A virus nucleoprotein epitope. J. Immunol. **151:**2658–2666.
4. **Doherty, P. C., W. Allan, M. Eichelberger, and S. R. Carding.** 1991. Heat-shock proteins and the γδ T cell response in virus infections: implications for autoimmunity. Semin. Immunopathol. **13:**11–25.
5. **Eichelberger, M., W. Allan, S. R. Carding, K. Bottomly, and P. C. Doherty.** 1991. Activation status of the CD4$^-$8$^-$ γδ-T cells recovered from mice with influenza pneumonia. J. Immunol. **147:**2069–2074.
6. **Fu, Y., R. Cranfill, M. Vollmer, R. van der Zee, R. O'Brien, and W. Born.** 1993. In vivo response of murine γδ T cells to a heat shock protein-derived peptide. Proc. Natl. Acad. Sci. USA **90:**322–326.
7. **Fu, Y., M. Vollmer, H. Kalataradi, K. Heyborne, C. Reardon, C. Miles, R. O'Brien, and W. Born.** 1994. Structural requirements for peptides that stimulate a subset of γδ T cells. J. Immunol. **152:**1578–1588.
8. **Gillis, S., and K. A. Smith.** 1977. Longterm culture of tumor-specific cytotoxic T cells. Nature (London) **268:**154–156.
9. **Haas, W., P. Pereira, and S. Tonegawa.** 1993. Gamma/delta cells. Annu. Rev. Immunol. **11:**637–685.
10. **Happ, M. P., R. T. Kubo, E. Palmer, W. K. Born, and R. L. O'Brien.** 1989. Limited receptor repertoire in a mycobacteria-reactive subset of γδ T lymphocytes. Nature (London) **342:**696–698.
11. **Hou, S., and P. C. Doherty.** 1993. Partitioning of responder CD8$^+$ T cells in lymph node and lung of mice with Sendai virus pneumonia by LECAM-1 and CD45RB phenotype. J. Immunol. **150:**5494–5500.
12. **Jindal, S., and R. A. Young.** 1992. Vaccinia virus infection induces a stress response that leads to association of Hsp70 with viral proteins. J. Virol. **66:**5357–5363.
13. **Johnson, R. M., D. W. Lancki, A. I. Sperling, R. F. Dick, P. G. Spear, F. W. Fitch, and J. A. Bluestone.** 1992. A murine CD4−, CD8−, T cell receptor-γδ lymphocyte clone specific for herpes simplex virus glycoprotein. J. Immunol. **148:**983–988.
14. **Kao, H. T., O. Capasso, N. Heintz, and J. R. Nevins.** 1985. Cell cycle control of the human HSP70 gene: implications for the role of a cellular E1A-like function. Mol. Cell. Biol. **5:**628–633.
15. **Kappler, J. W., B. Skidmore, J. White, and P. Marrack.** 1981. Antigen-inducible, H-2-restricted, interleukin-2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition. J. Exp. Med. **153:**1198–1214.
16. **Mombaerts, P., J. Arnoldi, F. Russ, S. Tonegawa, and S. H. Kaufmann.** 1993. Different roles of alpha beta and gamma delta T cells in immunity against an intracellular bacterial pathogen. Nature (London) **365:**53–56.
17. **Mombaerts, P., A. R. Clarke, M. A. Rudnicki, J. Iacomini, S. Itohara, J. J. Lafaille, L. Wang, Y. Ichikawa, R. Jaenisch, M. L. Hooper, and S. Tonegawa.** 1992. Mutations in T-cell receptor genes α and β block thymocyte development at different stages. Nature (London) **360:**225–231.
18. **O'Brien, R. L., M. P. Happ, A. Dallas, E. Palmer, R. Kubo, and W. K. Born.** 1989. Stimulation of a major subset of lymphocytes expressing T cell receptor γδ by an antigen derived from mycobacterium tuberculosis. Cell **57:**667–674.
19. **Portoles, P., J. Rojo, A. Golby, M. Bonneville, S. Gromkowski, L. Greenbaum, C. A. Janeway, Jr., D. B. Murphy, and K. Bottomly.** 1989. Monoclonal antibodies to murine CD3ε define distinct epitopes, one of which may interact with CD4 during T cell activation. J. Immunol. **142:**4169–4175.
20. **Roberson, S. M., and W. S. Walker.** 1988. Immortalization of cloned mouse splenic macrophages with a retrovirus containing the v-raf/mil and v-myc oncogenes. Cell. Immunol. **116:**341–351.
21. **Sciammas, R., R. M. Johnson, A. I. Sperling, W. Brady, P. S. Linsley, P. G. Spear, F. W. Fitch, and J. A. Bluestone.** 1994. Unique antigen recognition by a herpes-virus-specific TCR-gamma delta cell. J. Immunol. **152:**5392–5397.
22. **Sklar, M. D., A. Tereba, B. D. M. Chen, and W. S. Walker.** 1985. Transformation of mouse bone marrow cells by transfection with a human oncogene related to c-myc is associated with the endogenous production of macrophage colony stimulating factor-1. J. Cell. Physiol. **125:**403–412.
23. **Smith, G. L., J. Z. Levin, P. Palese, and B. Moss.** 1987. Synthesis and cellular location of the ten influenza polypeptides individually expressed by recombinant vaccinia viruses. Virology **160:**336–345.
24. **Tabi, Z., J. E. Allan, R. Ceredig, and P. C. Doherty.** 1988. Lethal vaccinia infection in cyclophosphamide-suppressed mice is associated with decreased expression of Thy-1, Lyt-2 and L3T4 and diminished IL-2 production in surviving T cells. Immunology **63:**423–429.
25. **White, J., M. Blackman, J. Bill, J. Kappler, P. Marrack, D. P. Gold, and W. Born.** 1989. Two better cell lines for making hybridomas expressing specific T cell receptors. J. Immunol. **143:**1822–1825.
26. **Wilson, C. M., J. W. Gatewood, J. M. McCormack, and W. S. Walker.** 1991. Immortalization of growth factor-dependent mouse splenic macrophages derived from cloned progenitors. J. Immunol. Methods **137:**17–25.