Activation of γδ T Cells in the Primary Immune Response to *Mycobacterium tuberculosis*

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Although the immunologic role of T cells bearing the conventional αβ T cell receptor (TCR) has been well characterized, little is known about the function of the population of T cells bearing the γδ TCR. Therefore, the role of γδ T cells in the immune response to *Mycobacterium tuberculosis* (MT) was investigated. The number of TCR γδ cells in the draining lymph nodes of mice immunized with MT was greatly increased in comparison with the number of TCR αβ cells. Three biochemically distinct γδ TCRs were detected. Analyses of cell cycle, of interleukin-2 receptor expression, and of interleukin-2 responsiveness showed that a large proportion of the γδ T cells were activated in vivo. TCR γδ cells responded to solubilized MT antigens in vitro but, in contrast to MT-specific αβ T cells, the response of γδ T cells to MT did not require major histocompatability complex class II recognition. These results provide an example of antigen-specific activation of γδ T cells in vivo and indicate that γδ T cells may have a distinct role in generating a primary immune response to certain microorganisms.

ALTHOUGH MOST T CELLS EXPRESS the αβ TCR (*1*), the γδ TCR is normally expressed on a small percentage of thymocytes and peripheral lymphocytes (*2, 3*). Like the α and β chains, diversity among the γ and δ chains is generated from the combinatorial rearrangement of different variable (V), diversity (D), and joining (J) segments to a constant (C) segment (*4*). Furthermore, the contribution of N-region diversity (partiularly in the δ chain) greatly expands the potential γδ repertoire (*5*). An important difference between γδ and αβ T cells is that most γδ T cells do not express the CD4 or CD8 cell surface molecules that participate in major histocompatibility complex (MHC) recognition by TCR αβ.

T cells expressing γδ TCRs appear to represent a separate T cell lineage from T cells expressing αβ TCRs (*2, 3*). Most γδ T cells develop within the thymus and are exported to the peripheral lymphoid organs (*6*). In the mouse, distinct epithelial compartments contain γδ T cells expressing different Vγ and Vδ products (*7*). Although γδ T cells can produce lymphokines (IL-2, IL-4, and γ-IFN) and can mediate cytolysis (*2*), effector functions thought to be critical in the generation of an immunologic response, a specific role for γδ T cells in the physiologic immune response has yet to be defined.

Intrigued by a report of increased numbers of $CD4^-CD8^-$ T cells in a tuberculous pleural effusion (*8*), we investigated the possible role of γδ T cells in the primary response to *Mycobacterium tuberculosis* (MT). B10.A mice were immunized with MT in the front and hind limbs, and the draining axillary and popliteal lymph nodes were collected. The B cell–depleted lymphocytes were analyzed for the presence of $CD3^+CD4^-CD8^-$ cells by staining with a monoclonal antibody (MAb) to CD3 (green) and MAbs to CD4 plus CD8 (red) and analyzing with two-color flow cytometry (FC). The percentage of $CD3^+CD4^-CD8^-$ T cells increased substantially after priming, whereas the percentages of $CD4^+$ and $CD8^+$ T cells did not change significantly (Fig. 1, A to C). Because there is a population of $CD3^+CD4^-CD8^-$ thymocytes bearing TCR αβ (*9*), we also examined the lymph nodes for expression of the $V_\beta 8$ products. In the thymus, more than 50% of the $CD4^-CD8^-$ TCR αβ thymocytes express V_β 8 (*9*). However, there were almost no $CD4^-CD8^-$ $V_\beta 8^+$ cells in the lymph nodes, either before or after priming (Fig. 1, D to F).

Although the percentages of $CD4^+CD8^-$, $CD4^-CD8^+$, and $CD4^-CD8^-$ $V_\beta 8^+$ populations show little change after priming, the $CD3^+CD4^-CD8^-$ $V_\beta 8^-$ population increases from 1.5% of $CD3^+$ cells in the unprimed lymph nodes to an average of 10.4% of $CD3^+$ cells 7 to 9 days after immunization. If the increase in total $CD3^+$ cells after priming is taken into account, there is a 20- to 25-fold increase in the absolute number of $CD3^+CD4^-CD8^-$ $V_\beta 8^-$ lymphocytes (Table 1). Furthermore, the expansion of this subset is dependent on the presence of MT, since injection of incomplete Freund's adjuvant alone did not increase the percentage of $CD3^+CD4^-CD8^-$ cells (1.6% of $CD3^+$ cells).

To confirm directly that the $CD3^+CD4^-CD8^-$ subset from the MT-primed lymph nodes predominantly expresses TCR γδ, we immunoprecipitated their CD3-associated receptors with antibodies to CD3 and to the γ chain and separated them by two-dimensional SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (first dimension nonreducing, second dimension reducing). Immunoprecipitation with the antibody to CD3 (Fig. 2A) shows three pairs of disulfide-linked proteins associated with the CD3 complex, and immunoprecipitation with the antiserum to Cγ1 plus Cγ2 shows that two of the three heterodimers (32 and 43 kD; 35 and 45 kD) contain products of the $C_\gamma 1$ or $C_\gamma 2$ genes. The known molecular

Table 1. Changes in lymph node T cell populations after priming with MT. The change in absolute cell number for the populations shown was calculated as (percentage of cells at 7 to 9 days × number of cells per lymph node at 7 to 9 days)/(percentage of cells in unprimed lymph nodes × number of cells per node in unprimed lymph nodes). The mean number of cells per unprimed lymph node was 2.1×10^6; the mean number of cells per lymph node at 7 to 9 days after priming was 1.25×10^7 cells. For DNA analysis of $CD4^+$ and $CD8^+$ lymphocytes, cells from draining lymph nodes were treated with RA3-3A1 plus complement as in Fig. 1, and dead cells were removed with two Ficoll gradients. Cells were stained with biotinylated antibody to CD4 (anti-CD4) or biotinylated antibody to CD8 and then FITC-labeled avidin. Cells were fixed in 1% paraformaldehyde for 20 min and incubated in 1% NP40 in phosphate-buffered saline (PBS) for 10 min and treated with ribonuclease A (100 U/ml) for 10 min at 37°C. Cells were washed twice in PBS and suspended in PBS plus propidium iodide (25 μg/ml). Cells were run on a FACSCAN with log amplification for FL1 (green fluorescence) and linear amplification for FL2 (propidium iodide fluorescence). $CD3^+4^-8^-$ $V_\beta 8^-$ lymphocytes were analyzed by treating draining lymph nodes with RA3-3A1 plus RL172 (anti-CD4) plus 83-12.5 (anti-CD8) plus complement as in Fig. 2. After removal of dead cells on two Ficoll gradients, cells were <1% $V_\beta 8^+$, $CD4^+$, or $CD8^+$. They were stained with MAb to CD3 plus FITC-labeled goat antibody to hamster immunoglobulin G, followed by fixation and propidium iodide staining as described above.

Cell population	Increase in absolute cell number at 7 to 9 days	>2N DNA at 5 days (%)
$CD4^+$	2.6-fold ($\times/\div$ 1.07)	2.5 ± 0.4
$CD8^+$	3.3-fold ($\times/\div$ 1.06)	2.2 ± 0.2
$CD3^+CD4^-CD8^-$ $V_\beta 8^-$	23.6-fold ($\times/\div$ 1.40)	18.8 ± 1.6

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weights of the γ and δ gene products and biochemical studies of $CD3^+CD4^-CD8^-$ thymocytes and splenocytes indicate that the lower molecular weight species of each pair represents the γ chain and the higher molecular weight species the δ chain (*2, 3*). Immunoprecipitation with the antiserum to $C_\gamma4$ demonstrates that the third disulfide-linked heterodimer (40 and 45 kD) contains the product of the $C_\gamma4$ gene. Different patterns of TCR γδ expression have been seen in different lymphoid as well as epithelial compartments. TCR γδ thymocytes express predominantly $V_\gamma2C_\gamma1$ gene products (*3*), whereas γδ T cells in the spleen express $V_\gamma1.1C_\gamma4$ in addition to $V_\gamma2C_\gamma1$ gene products (*10*). The pattern of expression of γδ T cells in MT-primed lymph nodes includes yet a third biochemically distinct TCR γδ reactive with the antiserum to $C_\gamma1$ plus $C_\gamma2$.

The increase in γδ T cells in response to MT priming suggests that they have been activated in vivo. To obtain more direct evidence for activation of these cells, we performed cell cycle analysis and examined them for induction of the IL-2 receptor (IL-2R), which normally becomes expressed on the surface of T cells in response to TCR occupancy (*11*). Propidium iodide DNA staining of MT-primed lymph node T cells shows that about 20% of the $\gamma\delta^+$ lymphocytes have >2N DNA content as compared with fewer than 5% for the $\alpha\beta^+$ ($CD4^+$ or $CD8^+$) lymphocytes (Table 1). Thus, the expansion of the $\gamma\delta^+$ population in response to MT priming appears to be the consequence of a high proliferative rate within the lymph node rather than preferential homing or trapping of nondividing γδ T cells.

Flow cytometric analysis of IL-2R expression by the different T cell populations from MT-primed lymph nodes revealed that a large proportion of $CD4^-CD8^-$ γδ T cells expressed IL-2R after immunization (12% high density IL-2R as opposed to 6% for $CD4^+CD8^-$ T cells and <1% for $CD4^-CD8^+$ T cells). It is important to assess the functional status of the IL-2Rs expressed on γδ T cells, since high affinity IL-2 binding and subsequent signal transduction requires expression of both the 55-kD and 70-kD IL-2R chains (*12*). Freshly isolated γδ T cells from MT-primed lymph nodes had a tenfold greater proliferative response to IL-2 than did unfractionated lymph node T cells (Table 2). Indeed, normalizing for the presence of γδ T cells within the total T cell population, we calculate that on day 3 roughly 50% of the total proliferative response of MT-primed lymph node T cells to IL-2 can be accounted for by the γδ T cell population.

To determine whether the in vivo activation of γδ T cells was in direct response to mycobacterial antigens, we tested for specific stimulation in vitro. In response to solubilized MT antigens, purified γδ T cells produce IL-2, as assayed on IL-2–dependent CTLL cells, and proliferate in an autocrine fashion (Tables 3 and 4). Purity of the responding γδ populations was confirmed by the absence of staining or stimulation by antibody to $V_\beta8$ and staining by a MAb directed against the αβ TCR (*13*).

The MT response of γδ T cells as opposed

Table 2. Proliferative response of T cell subsets from MT-primed lymph nodes to IL-2. Purified unfractionated or $CD3^+CD4^-CD8^-$ γδ T cells, prepared as described in Fig. 1 and Table 1, were incubated in 96-well plates (either 3,000 cells per well or 25,000 cells per well) with recombinant IL-2 (Cetus) (50 U/ml) for 2 days, labeled for 12 hours with [^{3}H]thymidine and collected to measure incorporation into DNA.

Cell population	Cells per well	[^{3}H]Thymidine incorporated
Unfractionated T cells	3,000	490 ±50
γδ T cells	3,000	5,440 ±420
Unfractionated T cells	25,000	6,960 ±300
γδ T cells	25,000	60,280 ±2,250

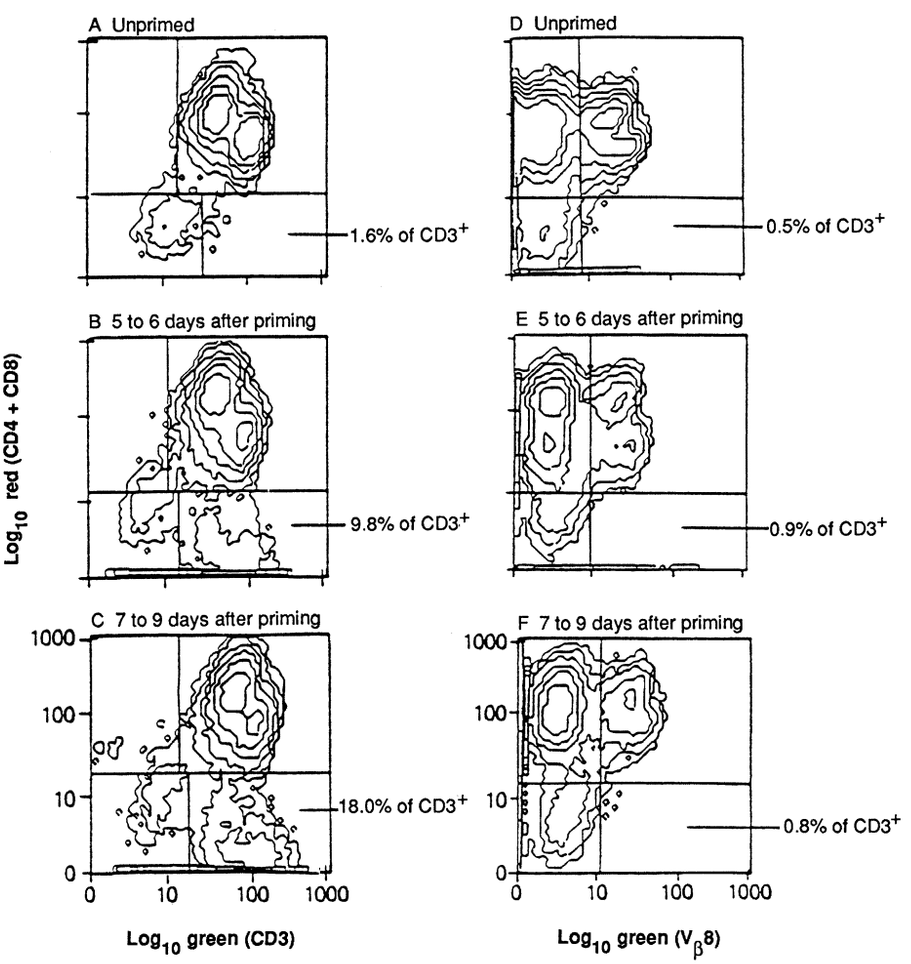

Fig. 1. Flow cytometric analysis of T cell subsets in draining lymph nodes after MT immunization. B10.A mice were immunized in the front and hind limbs with MT (strain H37Ra) in incomplete Freund's adjuvant (IFA). Draining popliteal and axillary lymph nodes from two to four mice were collected at (A and D) 3 days, (B and E) 5 to 6 days, or (C and F) 7 to 9 days after immunization and enriched for T cells by cytotoxic elimination of B cells with hybridoma RA3-3A1 (expressing MAb B220 directed against B cells) (*20*) plus complement. The remaining cells (90% to 95% $CD3^+$) were stained green with either (**A** to **C**) a mouse MAb to CD3 or (**D** to **F**) a MAb to $V_\beta8$, followed by MAbs to CD4 and CD8 (red). Staining combinations were as follows: (A to C) MAb to CD3 (145-2C11) (*21*). Fluorescein isothiocyanate (FITC)–labeled goat antibody to hamster immunoglobulin G (Kirkegard and Perry), biotinylated antibody to Lyt-2 (Becton Dickinson) plus biotinylated antibody to CD4 (H129.19) (*22*), and allophycocyanin-labeled avidin; (D to F) MAb to $V_\beta8$ (F23.1) (*23*), FITC-labeled goat antibody to mouse IgG2a (Southern Biotechnology), biotinylated antibody to Lyt-2 plus biotinylated antibody to CD4 (H129.19), and allophycocyanin-labeled avidin (Caltag). Cells (100,000 per sample) were analyzed on a Becton Dickinson 440 flow cytometer as described (*3*).

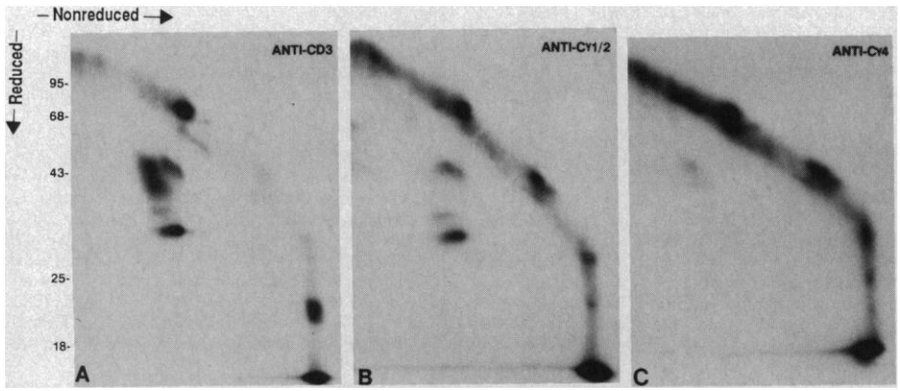

Fig. 2. Biochemical analysis of T cell receptors expressed by $CD4^-CD8^-$ T cells from MT-primed lymph nodes. $CD3^+CD4^-CD8^-$ cells were isolated from draining lymph nodes 7 days after MT priming by treatment with hybridomas RA3-3A1 (MAb 220 against B cells) RL172 (MAb against CD4) (*24*), and 83-12.5 (MAb against CD8.2) (*25*) plus complement. The remaining viable cells were isolated on Ficoll gradients, iodinated with ^{125}I by the lactoperoxidase method, and lysed in either 1% digitonin or 0.5% NP40 buffer. Digitonin lysates were immunoprecipitated with (**A**) MAb 145-2C11 against CD3, and NP40 lysates were immunoprecipitated with (**B**) the antiserum to $C_\gamma1$ and $C_\gamma2$, or (**C**) the antiserum to $C_\gamma4$. Immunoprecipitates were run on two-dimensional SDS-PAGE gels, with the first dimension run under nonreducing conditions (7.5% SDS-PAGE) and the second dimension under reducing conditions (10% SDS-PAGE). Gels were dried and exposed to Kodak XAR-5 film. Disulfide-linked T cell receptors were seen below the diagonal. Antisera, radioiodination, cell lysis, immunoprecipitation, and diagonal gels were as previously described (*3*).

Table 3. The IL-2–responsive line CTLL-2 was used as a measure of the amount of IL-2 produced in response to antigen stimulation. Supernatant (50 μl) was removed from T cell cultures 36 to 48 hours after antigen stimulation and was added to 3×10^3 CTLL cells. The CTLL cells were cultured for 24 hours, with [^{3}H]thymidine added for 12 hours. Duplicate wells were set up for all conditions and experiments. In all cases, standard errors of the mean were <11%. Media control backgrounds were 800 cpm for experiment 1, 1500 cpm for experiment 2, and 1100 cpm for experiment 3.

Cell population	Antigen	[^{3}H]Thymidine incorporated (cpm)		
		Experiment 1	Experiment 2	Experiment 3
Unfractionated T cells		810	1,620	1,280
	MT	28,020	19,700	30,160
γδ T cells		1,010	2,440	2,130
	MT	18,930	32,690	64,680

to that of unfractionated lymph node T cells was completely resistant to blockade with antibody to CD4. Since the CD4 molecule participates in MHC class II–restricted antigen recognition by TCR αβ (*14*), we investigated the possibility that MHC class II proteins have a role in antigen recognition by γδ T cells, which do not express CD4. Although a MAb to a class II MHC protein blocked most of the response of unfractionated day 8 lymph node T cells to MT, purified γδ T cells were not at all blocked (Table 4). In fact, the residual 10% to 15% stimulation of unfractionated T cells not blocked by the antibody to the MHC class II protein is likely accounted for by the approximately 10% γδ T cells. These data indicate that, in contrast to MT-reactive αβ T cells, MT-reactive γδ T cells do not require MHC class II molecules for the recognition of soluble antigens (*14, 15*). The γδ T cells were blocked by a $F(ab)_2$ antibody to CD3, an indication that the response of these cells was mediated by TCR recognition.

These results provide evidence that MT-specific γδ-expressing lymphocytes are activated in vivo and participate in the primary immune response to MT. It is unlikely that the expansion of the γδ T cell population is due to nonspecific proliferation of $IL\text{-}2R^+$ cells in response to IL-2 produced by αβ T cells, since purified γδ T cells both produce IL-2 and demonstrate autocrine proliferation in vitro in response to MT antigens. Furthermore, mice depleted of αβ T cells by in vivo treatment with antibodies to CD4 and CD8 show a similar 20- to 25-fold

Table 4. In vitro stimulation of γδ T cells from MT-primed lymph nodes. Unfractionated T cells and γδ T cells were isolated from MT-primted lymph nodes at 7 to 9 days after immunization and stimulated in vitro with soluble MT extract. Results of individual experiments are given as stimulation index (mean counts per minute with antigen/mean counts per minute without antigen). Values for the Δcpm, the difference between stimulated and unstimulated wells, are shown in parentheses (*27*). Unfractionated T cells were isolated by plating on duplicate plates coated with antibody to mouse Ig and recovering residual nonadherent cells. Purified γδ T cells were prepared as described in Fig. 2. Purified γδ T cell preparations were >90% $CD3^+$ and demonstrated no detectable staining with antibodies to $V_\beta8$ (F23.1), to CD4, to CD8, or to TCR αβ (*13*). Cells (2×10^5 in experiments 1 and 2 or 4×10^5 in experiments 3 and 4) were incubated with 2×10^5 irradiated (3000 R) B10.A splenocytes in 96-well plates for 3 days and then labeled with [^{3}H]thymidine for 12 hours and collected. A soluble extract of heat-killed, sonicated MT (strain H37Ra) at 10 μg per milliliter of protein was added as antigen where indicated. Anti-CD4(GK1.5) (*14*), anti-CD8(2.43) (*21*), and anti-MHC class II(Y17 + 10-2-16) (*28*) were added as a 1/100 dilution of ammonium sulfate–cut ascites. Anti-$V_\beta8$(F23.1) was added as a 1/10 dilution of culture supernatant. Anti-CD3(145-2C11) $F(ab)_2$ was purified over a protein A column after papain digestion and added at 50 μg/ml. Duplicate wells were set up for all conditions and experiments. In all cases, standard errors of the mean were <16%.

Cell population	Antigen	Antibody	Stimulation index (Δcpm)			
			Experiment 1	Experiment 2	Experiment 3	Experiment 4
Unfractionated T cells	MT	None	13.2 (23,670)	15.6 (41,450)	12.6 (91,780)	16.6 (69,320)
	MT	Anti-CD4	2.4 (1,610)			
	MT	Anti-CD4/CD8	2.3 (1,570)	2.6 (2,520)		
	MT	Anti-MHC class II			2.2 (9,350)	2.5 (6,530)
	MT	Anti-CD3 $F(ab)_2$				1.9 (3,980)
	None	F23.1	25.2 (46,930)	19.5 (52,450)		
γδ T cells	MT	None	3.8 (9,980)	5.3 (39,490)	7.0 (97,200)	9.9 (80,710)
	MT	Anti-CD4	4.1 (10,860)			
	MT	Anti-CD4/CD8	3.6 (8,020)	5.7 (29,980)		
	MT	Anti-MHC class II			6.4 (87,930)	9.3 (75,760)
	MT	Anti-CD3 $F(ab)_2$				1.5 (4,880)
	None	F23.1	1.1 (360)			

expansion of γδ T cells in response to MT-priming (*16*).

By all criteria of activation—high density IL-2R expression, response to IL-2, and in situ proliferation—the γδ T cell subset appears to be roughly ten times as active as the αβ T cell subset in the primary response to MT. This finding, together with the large amounts of IL-2 produced by isolated γδ T cells in response to MT stimulation, indicates that γδ T cells are important in generating lymphokines necessary for initiating the immune response to MT. An intriguing hypothesis is that γδ T cells have been evolutionarily selected to respond to certain common microbial antigens, thus enabling this population to respond quickly while the population of antigen-specific αβ T cells begins to expand. The secondary response may involve a switch in receptor usage (γδ early in the response, then αβ eventually taking over). Consistent with this notion is the virtual absence of expansion of the γδ T cell population after secondary MT immunization (*17*).

Among the many potential mycobacterial antigens, certain proteins have been identified as common to all the mycobacterial species and, furthermore, are homologous to various *Escherichia coli* heat shock proteins. A number of these proteins are important in the cell-mediated response to mycobacteria (*18*). A panel of unselected thymocyte TCR γδ hybridomas has been found to react with the 65-kD mycobacterial heat shock protein (*19*).

The failure of antibodies to MHC class II proteins to block the MT response of γδ T cells contrasts with the response of αβ T cells, whose recognition of soluble exogenous antigens (as confirmed in this study) generally requires MHC class II presentation. This result indicates that γδ T cells and αβ T cells recognize antigen differently and implies that they have distinct roles in the primary immune response. It will be of interest to determine the in vivo responses of γδ T cells to other bacterial pathogens.

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Neural Integration of Information Specifying Structure from Stereopsis and Motion

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When one views a two-dimensional parallel projection of dots on the surface of a rotating globe, the direction of rotation is ambiguous, and the perceived direction of rotation of the two-dimensional figure is unstable over time. Stability can be temporarily induced by adaptation to a three-dimensional globe with a direction of rotation unambiguously specified by stereo disparity; adaptation causes the two-dimensional figure to appear to rotate in the direction opposite that experienced during stereoscopic adaptation. This adaptation effect is selective for axis of rotation but is not shape-specific. It does depend on simultaneous stimulation by multiple depth planes defined by elements moving in different directions. Evidently information about stereopsis and information about structure from motion are integrated within a common neural site in the brain.

THE HUMAN VISUAL SYSTEM HAS SEVeral sources of information for recovering three-dimensional (3D) shape from two-dimensional (2D) images. One source is stereopsis, which arises from slight horizontal disparities between the two eyes' views. Indeed, so effective is stereopsis that it can extract depth and shape information from monocular images entirely devoid of recognizable form (*1*). A comparably effective source of information about 3D structure is relative motion (*2*). An object that has a shape that is ill-defined when stationary can be immediately perceived when that object moves (*3*). Both stereopsis and relative motion rely on multiple views of a scene to recover 3D structure; in the case of stereopsis the multiple views are provided by the two retinal images, whereas with relative motion the views are integrated over time. It has been proposed that stereopsis and relative motion are processed in common visual pathways (*4*). Here we report direct evidence that the visual nervous system integrates retinal disparity and relative motion information in the processing of 3D shape.

When one views a 2D parallel projection of dots on the surface of an object rotating about an axis, the direction of rotation is ambiguous; for instance, when rotation is about the vertical axis, observers experience a bistable percept whereby the figure's direction of rotation alternates over time between clockwise (CW) (that is, dots on the front surface move from right to left) and coun-

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