(AIDS) (5). We therefore suggest that progression of disease in HIV-infected individuals is not due to a switch from the T_H1 to the T_H2 phenotype, but may be favored by high and continuous HIV replication in $CD4^+$ T cells activated in vivo in response to the sustained production of T_H2-type cytokines (for example, through stimulation by common environmental allergens or helminthic infections). In contrast, some immunologic mechanism, such as activation of programmed cell death after gp120-CD4 interaction or mediated by an HIV-associated superantigen (16), may be responsible for the depletion or functional impairment (or both) of T_H1-type $CD4^+$ T cells, as observed even at the clonal level in patients with full-blown AIDS and repeated opportunistic infections (8). Thus, understanding the reasons for the selective replication of HIV may be of therapeutic value in the treatment of HIV-infected individuals.

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Lack of Evidence for the Dichotomy of T_H1 and T_H2 Predominance in HIV-Infected Individuals

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A switch from a T helper 1 (T_H1) cytokine phenotype to a T_H2 phenotype has been proposed as a critical element in the progression of human immunodeficiency virus (HIV) disease. Here, constitutive cytokine expression was analyzed in unfractionated and sorted cell populations isolated from peripheral blood and lymph nodes of HIV-infected individuals at different stages of disease. Expression of interleukin-2 (IL-2) and IL-4 was barely detectable (or undetectable) regardless of the stage of disease. $CD8^+$ cells expressed large amounts of interferon γ and IL-10, and the levels of these cytokines remained stably high throughout the course of infection. Furthermore, similar patterns of cytokine expression were observed after stimulation in vitro of purified $CD4^+$ T cell populations obtained from HIV-infected individuals at different stages of disease. These results indicate that a switch from the T_H1 to the T_H2 cytokine phenotype does not occur during the progression of HIV disease.

Two populations of $CD4^+$ T_H lymphocytes have recently been identified in mice on the basis of their mutually exclusive production of certain cytokines such as IL-2 and interferon γ (IFN-γ) (T_H1 $CD4^+$ cells) or IL-4, IL-5, and IL-10 (T_H2 $CD4^+$ cells) (1). A similar dichotomy between T_H1 and T_H2 cells has been identified in human $CD4^+$ T lymphocytes (2); however, in contrast to mice IL-10 is produced by both T_H1 and T_H2 cells in humans (1–4). In certain infectious diseases in mice and humans, particularly parasitic diseases, the T_H1 pattern of cytokines is associated with resistance to infection (5), whereas the T_H2 pattern is associated with progressive forms of infection (5); however, association between the T_H2 pattern and protection has been demonstrated in malaria and in certain intestinal helminth infections (5). Recently, Clerici and Shearer have investigated whether a switch from the T_H1 to the T_H2 cytokine phenotype occurs during the course of HIV infection (6). They measured the production of IL-2, IL-4, and IL-10 after in vitro stimulation with recall antigens of unfractionated peripheral blood mononuclear cells collected from HIV-infected individuals in early stages of the disease and after stimulation with phytohemagglutinin (PHA) of cells collected from patients in intermediate and late stages of the disease (6). They reported that IL-2 production decreased and IL-4 and IL-10 production increased (6) with disease progression. On the basis of these findings, they proposed that a switch from the T_H1 (IL-2 and IFN-γ) to the T_H2 (IL-4 and IL-10) cytokine phenotype is a critical step in the progression of HIV disease (6).

To address the question of a switch from the T_H1 to the T_H2 cytokine phenotype in HIV infection, we undertook several experimental approaches. (i) We performed cross-sectional analysis of the constitutive expression of a group of cytokines (IL-2, IL-4, IL-10, and IFN-γ) in unfractionated mononuclear cells isolated from peripheral blood and lymph nodes from the same HIV-infected individuals in different stages of disease. The measurement of constitutive cytokine expression ex vivo may provide important information on the predominant pattern of cytokine expression in vivo and may avoid the variability and potential for artifact that is inherent in the in vitro stimulation of heterogeneous and functionally defective mononuclear cell populations (7). (ii) We performed longitudinal analysis of constitutive cytokine expression in peripheral blood mononuclear cell samples collected from the same patient at different

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time points during disease progression. Because of the extreme heterogeneity of the course of HIV infection among different HIV-infected individuals, this analysis offers an opportunity to observe changes in the pattern of cytokine expression occurring over time in the same individual. (iii) We analyzed constitutive cytokine expression in sorted T cell ($CD4^+$ and $CD8^+$) subsets. (iv) We analyzed cytokine expression after in vitro stimulation of purified $CD4^+$ T cells from HIV-infected individuals at different stages of disease. This analysis specifically addresses, under experimental conditions (in vitro cell activation) similar to those used by Clerici and Shearer, whether a switch from the T_H1 to the T_H2 cytokine pattern can be detected during disease progression.

Patients were divided empirically into three groups according to their absolute count of $CD4^+$ T cells (early stage disease, more than 500 $CD4^+$ T cells per microliter; intermediate stage disease, 200 to 500 $CD4^+$ T cells per microliter; late stage disease, less than 200 $CD4^+$ T cells per microliter). The amounts of cytokine expression in the two lymphoid compartments were determined by a semiquantitative polymerase chain reaction (PCR) assay (8, 9) with the use of a panel of primer pairs specific for each cytokine analyzed (*10*). To avoid the variability that could have been generated by analyzing the samples in separate PCR reactions, we assayed simultaneously samples from peripheral blood and lymph nodes for each cytokine. Cross-sectional analysis of ex vivo cytokine expression was performed in unfractionated mononuclear cells isolated from peripheral blood and lymph nodes of 14 HIV-infected individuals at different stages of disease and compared with that observed in peripheral blood (four donors) and lymph nodes (reactive lymph nodes obtained from five donors who underwent cardiac or biliary tract surgery) of HIV-negative individuals (Fig. 1). In peripheral blood, overall expression of IFN-γ and IL-10 was greater in HIV-infected individuals compared to that in HIV-negative individuals but did not change with disease progression (Fig. 1, B and D). Expression of IL-2 and IL-4 was barely detected or not detected in both HIV-infected and HIV-negative individuals (Fig. 1, A and C). In lymph nodes, expression of IFN-γ and IL-10 was greater in HIV-infected versus HIV-negative individuals and remained unchanged at different stages of disease (Fig. 1, B and D). In confirmation of previous studies (*11*), we have demonstrated that greater levels of expression of IFN-γ are associated with HIV infection. Expression of IL-2 and IL-4 in lymph nodes of HIV-infected individuals was generally very low at any stage of disease, which is similar to their expression in HIV-negative individuals (Fig. 1, A and C). Regardless of the stage of the disease, the patients who expressed detectable levels of IL-4 or IL-2 (1, 3, 9, 13, and particularly 12) also expressed IFN-γ and IL-10 (Fig. 1, B and D). Thus, our results do not demonstrate a switch from T_H1 to T_H2 in the pattern of in vivo constitutive expression of cytokines over the course of disease progression.

We further investigated this issue by performing longitudinal analyses of cytokine expression in peripheral blood mononuclear cell samples collected from the same patients at three different times during disease progression (Fig. 2). In the three patients studied (15, 16, and 17), the $CD4^+$ T cell count was 900 to 1000 per microliter at the time of collection of the first sample, 500 to 600 per microliter in the second sample, and 200 to 300 per microliter in the third sample. In all patients studied here, constitutive expression of IL-2 and IL-4 was barely detected or not detected at any time (Fig. 2, A and C). The results obtained for expression of IFN-γ and IL-10 were variable (Fig. 2, B and D). In patients 15 and 17, there were no substantial changes in expression of either IFN-γ or IL-10 over time, whereas in patient 16, a two- to threefold increase over time was observed for both IFN-γ and IL-10. Thus, longitudinal analysis of cytokine expression in the same patients likewise did not indicate a switch in cytokine profile expressed in early versus advanced stage disease. It is likely that the tendency for the concentration of IL-10 to increase in late stage disease may be the result of quantitative changes in the composition of cell subsets between the cell samples collected in early stage disease versus those collected in late stage disease

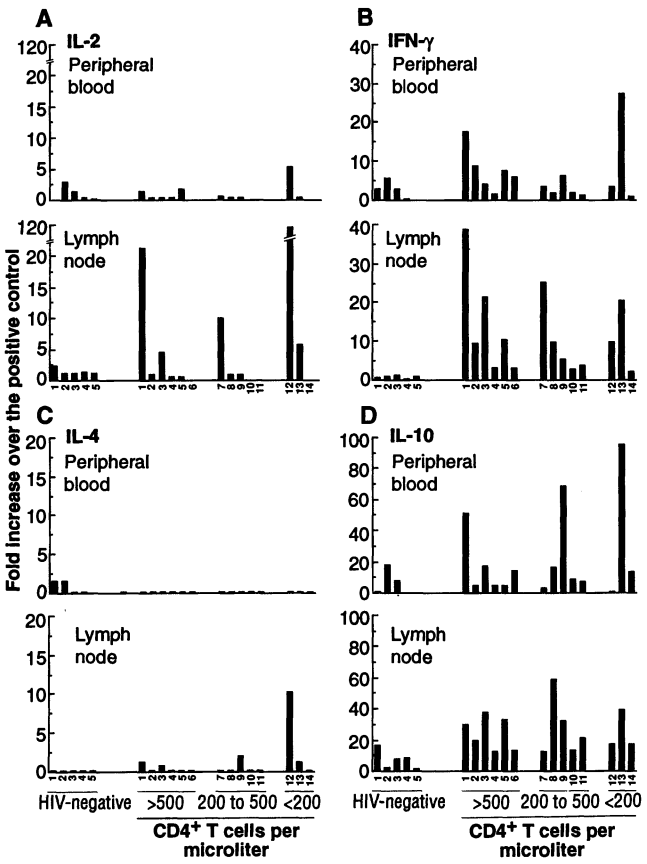

Fig. 1. Analysis of cytokine expression in peripheral blood and lymph nodes from HIV-negative versus HIV-infected individuals at different stages of disease. (**A**) IL-2 expression. (**B**) IFN-γ expression. (**C**) IL-4 expression. (**D**) IL-10 expression. Patients were divided into three groups (1 to 6, 7 to 11, and 12 to 14) according to their absolute count of $CD4^+$ T cells. Unfractionated mononuclear cells isolated from peripheral blood and lymph nodes from the same patient were pelleted in aliquots of 5×10^6 per vial and stored at −70°C until used. RNA extraction and PCR amplification were performed as described (*8, 9*). For each cytokine analyzed, samples obtained from peripheral blood and lymph nodes of HIV-infected individuals were amplified in the same PCR reaction. Results are expressed as the fold increase over the positive control (*8, 9*).

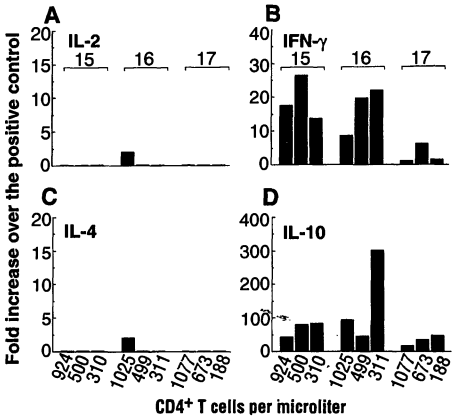

Fig. 2. Longitudinal analysis of cytokine expression during disease progression; shown is expression of IL-2 (**A**), IFN-γ (**B**), IL-4 (**C**), and IL-10 (**D**). Frozen samples of unfractionated peripheral blood mononuclear cells collected at three different time points during disease progression from the same patient were thawed, and cell pellets (5×10^6 cells per vial) were prepared for PCR that was performed as described (*8, 9*). Patient numbers are shown at the top of the figure.

rather than a qualitative functional switch of a particular cell subset. It is possible that the stably elevated expression of IL-10 may impair the functions of both T_H1 and T_H2 $CD4^+$ T cell subsets (*3, 12*) over the course of HIV disease.

To determine the cell types involved in the expression of these cytokines, we analyzed sorted T cell subsets. In a preliminary experiment, mononuclear cells isolated from peripheral blood and lymph node were stained with monoclonal antibody to CD4 and sorted into $CD4^+$ versus $CD4^-$ cell subsets. Expression of IFN-γ and IL-10 in both peripheral blood and lymph node was predominantly restricted to the $CD4^-$ cell subset, which may contain $CD8^+$ cells, monocytes, B cells, and natural killer (NK) cells (Fig. 3A). Constitutive levels of IL-2 and IL-4 were very low or not detected in any cell subset (Fig. 3A). We more precisely delineated the different cell subsets responsible for cytokine expression in the two lymphoid compartments by analyzing individually sorted T cell ($CD4^+$ and $CD8^+$) subsets in a patient (11) with 300 $CD4^+$ T cells per microliter. In patient 11, the $CD8^+$ cell subset was predominantly responsible for the expression of IFN-γ, and to a lesser extent of IL-10, in both peripheral blood and lymph node (Fig. 3B). In this patient, $CD4^+$ cells did not express significant amounts of any of the cytokines analyzed (Fig. 3B). The predominant expression of IFN-γ by $CD8^+$ T cells compared to that expressed by $CD4^+$ T cells was further confirmed in sorted cell populations isolated from the lymph node of another patient (18, who had an absolute $CD4^+$ T cell count of 517 per microliter). The levels of IFN-γ expression were more than 10-fold greater in the $CD8^+$ cells compared to those in the $CD4^+$ cells (68- versus 5-fold increase over the positive control).

Cytokine expression was also determined in sorted or purified (>95%) peripheral blood $CD4^+$ T cells from two HIV-negative individuals. Expression of IL-2, IL-4, IL-10, and IFN-γ was very low and similar to that observed in sorted $CD4^+$ T cells in patients 6 and 11 (Fig. 3) (*13*). The small amount of constitutive cytokine expression in $CD4^+$ T cells from HIV-negative individuals is consistent with the absence of cell activation.

The observations that constitutive expression of cytokines by $CD4^+$ T cells is very low (Fig. 3) and that IL-2 and IL-4, which are both $CD4^+$ T cell–dependent cytokines, are generally barely detected cannot be reconciled with the fact that many $CD4^+$ T lymphocytes isolated from lymph nodes express HLA-DR (*14*), a marker of activation on human T cells (*15*). The mean percentage of $CD4^+$ T cells that expressed DR in the lymph nodes of seven patients included in our study was 36.9 ± 13.2, whereas in the same lymph nodes the mean percentage of $CD8^+$ T cells that expressed DR was 44.7 ± 21.3. The discrepancy between the low levels of constitutive cytokine expression by $CD4^+$ T cells (Fig. 3) and the state of activation of these cells represents a major difference between $CD4^+$ and $CD8^+$ T cells. In contrast to $CD4^+$ T cells, many $CD8^+$ T cells are activated (express HLA-DR) and in these cells high levels of constitutive cytokine expression, particularly that of IFN-γ, were observed (Fig. 3). This discrepancy may reflect a dysfunction of $CD4^+$ T cells in vivo, despite the fact that expression of all the cytokines analyzed in this study can be efficiently induced in purified $CD4^+$ T cell populations after stimulation in vitro.

The absence of constitutive expression of $CD4^+$ T cell–dependent cytokines, particularly IL-2, and the high levels of IFN-γ expression observed here may be relevant to the phenomenon of programmed cell death or apoptosis in HIV-infected individuals (*16*). It has recently been demonstrated in vitro that resting or activated $CD4^+$ and $CD8^+$ T cells as well as thymocytes from healthy HIV-negative individuals undergo apoptosis if they are stimulated through the T cell receptor complex in the absence of accessory cells (*17*). Apoptosis was demonstrated to be associated with IFN-γ expression in the absence of IL-2 expression (*17*), a situation analogous to our results. Hence, the cytokine profile

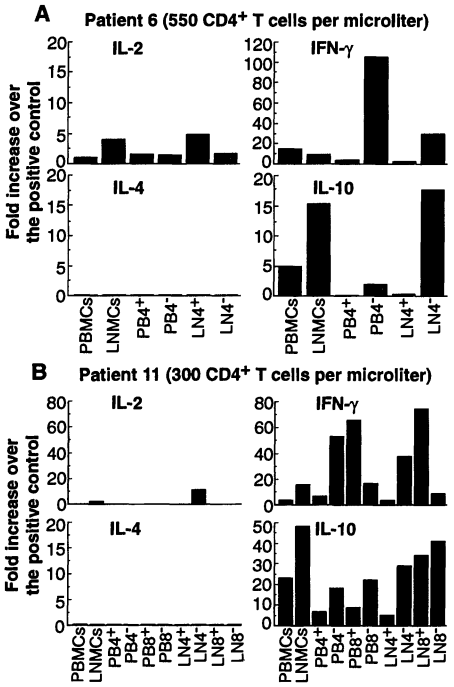

Fig. 3. Cytokine expression in sorted cell populations isolated from peripheral blood and lymph nodes from two HIV-infected individuals. (**A**) Cytokine expression (IL-2, IFN-γ, IL-4, and IL-10) in sorted $CD4^+$ and $CD4^-$ cell subsets isolated from peripheral blood (PB) and lymph node (LN) of patient 6. (**B**) Cytokine expression in sorted $CD4^+$, $CD4^-$, $CD8^+$, and $CD8^-$ cell subsets isolated from peripheral blood and lymph node of patient 11. Peripheral blood and lymph node mononuclear cells (LNMCs) were stained with either fluorescein isothiocyanate–conjugated CD4 or CD8 monoclonal antibody and sorted in $CD4^+$, $CD4^-$, $CD8^+$, and $CD8^-$ cell subsets. Cell sorting was performed as described (*24*). The degree of purity of the different sorted cell populations ranged from 96 to 98%. In both (A) and (B), for each cytokine analyzed, samples obtained from unfractionated and sorted cell populations were amplified in the same PCR reaction.

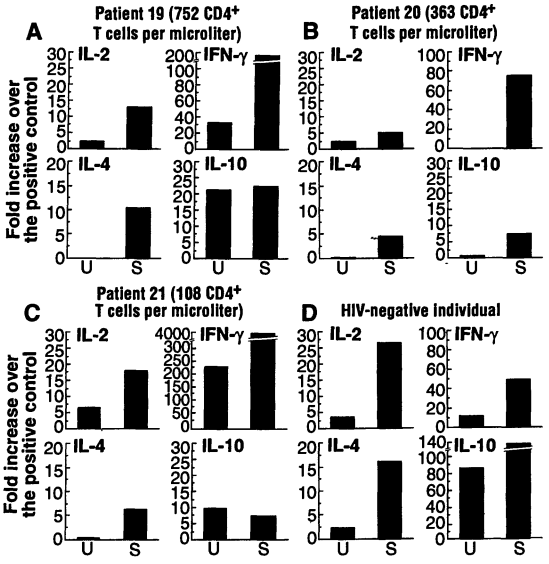

Fig. 4. Cytokine expression in purified $CD4^+$ T cell populations after in vitro stimulation. Isolation and stimulation of purified $CD4^+$ T cell populations were performed as described (*20*). $CD4^+$ cell populations were obtained from three HIV-positive individuals (**A** through **C**) at different stages of disease and from one HIV-negative donor (**D**). Cytokine expression was evaluated in unstimulated (U) $CD4^+$ cells and in $CD4^+$ cells stimulated (S) after cross-linking of the CD3 molecule. Data are expressed as the fold increase over the positive control (*8, 9*). Contaminating cell populations (monocytes and B cells) may have accounted, at least in part, for the amounts of IL-10 and IFN-γ observed. Because the percentage of $CD8^+$ T cells was less than 5% in the purified $CD4^+$ T cell populations, it is unlikely that these $CD8^+$ T cells contributed substantially to the increased levels of expression of IL-2 and IL-4 after cross-linking of CD3. Nonetheless, it cannot be definitely established that all of the IL-2 and IL-4 expression occurred in $CD4^+$ T cells.

described here may lead to a higher susceptibility to apoptosis in cells that have received aberrant activation signals during the course of HIV infection. Furthermore, it has been demonstrated that IFN-γ influences the balance of lymphocyte traffic between the blood and the lymphoid tissue by inducing retention of these cells within the lymphoid organs (*18*). Thus, the high expression of IFN-γ in both peripheral blood and lymph node that was observed in HIV-infected individuals (Figs. 1 and 2) may favor the retention of circulating lymphocytes within the lymph nodes and may contribute to the development of the lymphadenopathy associated with certain phases of HIV disease (*19*).

Finally, cytokine expression was evaluated in purified $CD4^+$ T cell populations after activation in vitro. $CD4^+$ T cells isolated from peripheral blood of three HIV-infected individuals in different stages of disease (patients 19, 20, and 21) were negatively selected by a panning procedure (*20*). The purity of the $CD4^+$ cell populations ranged between 80 to 90%; contaminating cells were $CD8^+$ T cells (<5%) and a mixture of B cells, monocytes, and NK cells. Cytokine expression was compared in unstimulated purified $CD4^+$ T cells and in purified $CD4^+$ T cells after stimulation by cross-linking of the CD3 molecule. In all three patients analyzed, IFN-γ expression was greatly increased after stimulation, regardless of the stage of disease (Fig. 4). Expression of IL-2 and IL-4 increased (2- to 10-fold), although this was less than the increase in expression of IFN-γ in stimulated purified $CD4^+$ T cell populations compared to that in unstimulated cell populations (Fig. 4). This increase in expression of IL-2, IL-4, and IFN-γ was consistently observed in the three patients and was not associated with any particular stage of disease (Fig. 4). Similar induction of IL-2, IL-4, and IFN-γ expression was observed in purified $CD4^+$ cell populations from an HIV-negative donor (Fig. 4). In both HIV-infected and HIV-negative individuals (Fig. 4), expression of IL-10 was not generally increased in stimulated $CD4^+$ cell populations compared to that in unstimulated $CD4^+$ cell populations. Only in patient 20 did IL-10 expression increase after stimulation, but overall the levels of induction were very low (Fig. 4).

We have found that both cross-sectional and longitudinal analyses of constitutive cytokine expression did not demonstrate a switch from the T_H1 to the T_H2 phenotype during disease progression in unfractionated or sorted $CD4^+$ cell populations isolated from peripheral blood and lymph node. In addition, no evidence of a switch in the cytokine pattern was detected after in vitro activation (Fig. 4). In other studies, the occurrence of the T_H1 to T_H2 switch has been investigated either in vitro (by analyzing cytokine production in a large number of clones obtained from HIV-infected individuals at different stages of disease) (*21*) or in vivo [by analyzing levels of immunoglobulin E (IgE) (*21, 22*) and soluble CD23 (*23*)]. A switch associated with disease progression could not be demonstrated by the study of cytokine production at the clonal level (*21*) or by the determination of soluble CD23 (*23*), although studies have suggested that a bias in cytokine production (*21, 22*) (increased amounts of IgE) may occur only in a few HIV-infected individuals in late stages of the disease. Therefore, taken together, our data do not support the hypothesis that a switch from T_H1 to T_H2 cytokine pattern is a critical step in the progression of HIV disease.

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(Synthecell Vega, Columbia, MD), 200 μM dNTPs (Boehringer Mannheim), and 2 U of Taq polymerase (Perkin-Elmer). An amount of antisense primer end-labeled with [γ-^{32}P]adenosine triphosphate (Amersham, Arlington Heights, IL) corresponding to 500,000 to 1,000,000 cpm/μl was also added. Amplification was performed in the GeneAmp PCR System 9600 (Perkin-Elmer Cetus) with the following incubation times: 30-s denaturation at 94°C (60 s for the first cycle only), 45-s annealing at 60°C (55°C for the Cα primer pair), and 60-s extension at 72°C. Products of amplification were analyzed by electrophoresis in 5%, 29:1 polyacrylamide gels and visualized by autoradiography. The intensity of the radioactive signal for each cytokine was measured with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Semiquantitative analysis was performed with a modification of the method described in (*9*). A simple regression curve was fitted for the twofold dilutions of the standard cDNA, and the equation obtained was used to determine the amount of target sequence in the patient samples. Results were expressed as fold increase over the positive control. The positive control corresponds to the last dilution (1:6400) of the standard cDNA in which amplified products (positive signal) may be detected.

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20. To obtain purified CD4$^+$ cell populations, we resuspended 15 × 10^7 PBMCs in phosphate-buffered saline (PBS; ×1) at 20 × 10^6 cells per milliliter, and monoclonal antibodies to the Fc receptor (1 μg per 10^6 cells), CD8 (2 μg per 10^6 cells), and CD19 (5 μg per 10^6 cells) were added at the appropriate concentrations. Cells were incubated in the presence of the different antibodies at 4°C for 45 min. At the end of the incubation, cells were washed three times in cold PBS, resuspended in cold PBS containing 2% fetal bovine serum (FBS) at 5 × 10^6 to 7 × 10^6 cells per milliliter, and 7.5 ml of the cell suspension was plated in petri dishes that were precoated with goat antibody to mouse (Sigma) and incubated at 4°C for 45 min. At the end of the incubation, cells were harvested (petri dishes were washed five times with 5 ml of PBS), washed, and resuspended in RPMI + 20% FBS at 10^7 cells per milliliter. After this negative selective procedure, the percentage of CD4$^+$ cells ranged between 80 and 90%. The contaminating cells included CD8$^+$ cells (<5%) monocytes, B cells, and NK cells. To perform in vitro stimulation, we incubated purified CD4$^+$ cell populations (5 × 10^6 cells per 0.5 ml) with purified antibody to CD3 (8 μg/ml) on ice for 60 min. Then, to induce cross-linking of the CD3 molecule, we added goat antibody to mouse (50 μg/ml) and incubated the cells at 37°C for 4 hours. At the end of the incubation, we prepared cell pellets for the analysis of cytokines by PCR and stored them at −80°C until they were used.

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The Thermal Grill Illusion: Unmasking the Burn of Cold Pain

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In Thunberg's thermal grill illusion, first demonstrated in 1896, a sensation of strong, often painful heat is elicited by touching interlaced warm and cool bars to the skin. Neurophysiological recordings from two classes of ascending spinothalamic tract neurons that are sensitive to innocuous or noxious cold showed differential responses to the grill. On the basis of these results, a simple model of central disinhibition, or unmasking, predicted a quantitative correspondence between grill-evoked pain and cold-evoked pain, which was verified psychophysically. This integration of pain and temperature can explain the thermal grill illusion and the burning sensation of cold pain and may also provide a basis for the cold-evoked, burning pain of the classic thalamic pain syndrome.

The sensations of pain and temperature stem from parallel ascending sensory channels that are regarded as physiologically separate (*1*). However, these sensations can be shown to interact. In 1896, Thunberg reported that innocuous warm and cool stimuli applied simultaneously to the skin by means of interlocking spiral tubes elicited a sensation of strong heat, which he compared to the burning sensation that commonly accompanies cold pain (*2*). We investigated the cause of this illusion with neurophysiological and psychophysical methods.

The prevailing explanation of the thermal grill illusion is based on Alrutz's proposal that the perception of "heat" (evoked at temperatures between 45° and 50°C) is not a specific sensation but rather a fusion resulting from the simultaneous activation of specific warm and cold spots (*3*). (Cold spots can be activated by cooling and also, paradoxically, by high temperatures.) The grill was thought to evoke this fusion by the simultaneous activation of sensory channels for warmth and cold by warm and cool, rather than hot, temperatures. In the 1920s, several experimental psychologists concurred with this proposal, whereas others concluded that the sensation evoked by the grill was more tactual, like a pricking sensation. One group questioned whether the illusion was due to suggestion and to the confusion resulting from an unnatural stimulus.

Modern physiological findings have confirmed the existence of specific cutaneous receptors for warm and for cold. However, many warm receptors cease their discharge at temperatures above 45°C and are thus not active at high temperatures (*1*). Instead, specific heat nociceptors have thresholds around 45°C, which is now the accepted threshold for perception of heat pain (*1, 4*). These findings contradict the fusion hypothesis, because nociceptors, but not warm receptors, are activated by high temperatures, whereas warm receptors, but not nociceptors, are activated by the grill.

We considered the alternative hypothesis that the grill illusion results from an unmasking rather than a fusion. Thunberg suggested that fusion could be shown if a selective block of the sensory channel for warmth enabled a hot stimulus to elicit a cold sensation (*2*), but in fact the converse occurs. Selective elimination of sensibility to cold, but not warmth (produced by a pressure block of peripheral, cold-specific, Aδ nerve fibers), actually enables a cold stimulus (at temperatures up to 25°C) to elicit a burning heat sensation (*5*). The primary afferents responsible are probably C polymodal nociceptors, many of which respond to cold as well as to heat and sometimes to pinch (*5, 6*). Thus, we hypothe-

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