

CD4-CD8 and CD28 Expression in T Cells Infiltrating the Vitreous Fluid in Patients With Proliferative Diabetic Retinopathy

A Flow Cytometric Analysis

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Objectives: To investigate CD4-CD8 and CD28 expression in T cells infiltrating the vitreous fluid in patients with proliferative diabetic retinopathy and to evaluate the relationship between the infiltrating T cells and both the activity of proliferative diabetic retinopathy and the clinical outcome.

Methods: Both vitreous and peripheral blood samples were obtained simultaneously from 20 consecutive diabetic patients and analyzed by flow cytometry. Three diabetic patients were excluded because there were no viable cells in the vitreous fluid. Six nondiabetic patients requiring vitrectomy were also studied.

Results: T lymphocytes were detected in all 6 diabetic patients with vitreous hemorrhage and in 6 (55%) of the 11 diabetic patients without vitreous hemorrhage, but in none of the nondiabetic patients. The percentages of T cells (CD3⁺), TCD4⁺ (CD3⁺ CD4⁺), and TCD8⁺ (CD3⁺ CD8⁺) subsets, as well as the expression of CD28, were similar in the vitreous fluid and in the peripheral blood in patients with vitreous hemorrhage. However, in pa-

tients without vitreous hemorrhage, the percentage of CD4⁺ CD28⁻ T cells in the vitreous fluid was significantly higher than in the peripheral blood (33.34% [20.75%-100.00%] vs 8.45% [2.43%-56.59%]; $P=.02$). In addition, all of these patients showed quiescent retinopathy and their outcome was better than that of patients with vitreous hemorrhage and patients in whom intra-vitreous T cells were undetectable.

Conclusion: T cells infiltrating the vitreous of diabetic patients without vitreous hemorrhage not only show a different pattern than in the peripheral blood but also seem to improve the prognosis of proliferative diabetic retinopathy.

Clinical Relevance: Our results provide further understanding of events involved in the autoimmune response in diabetic retinopathy and may aid in the research for new treatment approaches.

Arch Ophthalmol. 2004;122:743-749

PROLIFERATIVE DIABETIC RETINOPATHY (PDR) is a major cause of adult blindness, and it is characterized by the appearance of neovascularization. The new vessels are fragile and lack the normal barrier function, thereby permitting extravascular leakage of blood components. In the later stages, fibrovascular proliferative changes may result in vitreous hemorrhage or tractional retinal detachment that often requires surgical intervention. The precise mechanisms involved in the etiopathogenesis of PDR have not been elucidated, but retinal ischemia seems to be crucial in the angiogenic stimulus, thus determining the synthesis of several growth factors, such as vascular endothelial growth factor.^{1,2}

There is growing evidence that leukocytes are involved in capillary nonperfusion, retinal vascular leakage, and endo-

thelial cell damage in diabetic retinopathy.³⁻⁸ Moreover, leukocytes have a relevant role in the angiogenic and fibrotic processes that occur in PDR by means of the secretion of several cytokines and proteases. Several alterations in the properties of leukocytes have been reported in diabetes, such as decreased deformability,⁶ increased activation,³ and adhesiveness to vascular endothelium.⁴⁻⁸ In addition, the increased expression of adhesion molecules such as intercellular adhesion molecule 1^{8,9} has been found in retinal endothelial cells in diabetic retinopathy, facilitating retinal leukocyte stasis (leukostasis). Moreover, our group recently reported that cellular adhesion and angiogenesis may be linked processes in diabetic patients with PDR.¹⁰

Although there is much evidence that leukocytes participate in the etiopathogenesis of PDR, there is little information on the specific role of lymphocytes. The

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vitreous body is an immune-privileged site protected from systemic circulation by the blood-retinal barrier.^{11,12} The breakdown of the blood-retinal barrier is a characteristic feature of PDR and, in consequence, could facilitate the passage of immune cells from systemic circulation into the vitreous body. However, as far as we know, no studies have investigated T-lymphocyte subsets in the vitreous fluid of diabetic patients.

In the present study, we have flow cytometry to analyze vitreous fluid from patients with PDR for the presence of CD4⁺ (helper/inducer) and CD8⁺ (cytolytic/cytotoxic) T cells, as well as the expression of the costimulatory molecule CD28. In addition, these results were compared with those obtained in samples of peripheral blood from the same patients. Finally, we evaluated the relationship between the infiltrating T cells and both the activity of PDR and the clinical outcome.

METHODS

SUBJECTS

Twenty consecutive patients with type 2 diabetes mellitus (11 women and 9 men; mean \pm SD age, 62.82 ± 12.12 years) with PDR in whom a classic 3-port pars plana vitrectomy was performed were initially considered in this study. Tractional retinal detachment was the main reason for performing vitrectomy in these patients. Six nondiabetic patients (3 women and 3 men; mean age, 47.33 ± 33.50 years) requiring vitrectomy for macular holes ($n=3$) and subretinal membranes ($n=3$) were also studied. Patients who had undergone previous vitreoretinal surgery, had had a vitreous hemorrhage in the preceding 2 months, or had received photocoagulation in the previous 3 months were all excluded.

Retinopathy was graded intraoperatively by the same ophthalmologist, taking into consideration the presence of active neovascularization whenever perfused preretinal capillaries were found and quiescent retinopathy whenever nonperfused vessels or fibrosis was present.

After vitrectomy, an ophthalmologic evaluation was systematically performed each month for the first 3 months, and subsequently a bimonthly evaluation was conducted. The overall follow-up was 8.3 ± 2.5 months (range, 3-12 months).

SAMPLE COLLECTION AND IDENTIFICATION OF VITREOUS HEMORRHAGE

Both venous blood and vitreous samples were collected simultaneously at the time of vitreoretinal surgery. Undiluted vitreous samples (0.5-1 mL) were obtained at the onset of vitrectomy by aspiration into a 1-mL syringe attached to the vitreous cutter (Series Ten Thousand Ocutome; Alcon Laboratories, Irvine, Calif) before the intravitreal infusion of balanced saline solution was started. The vitreous samples were transferred to a tube and delivered to the laboratory at room temperature as rapidly as possible after collection, usually within 30 minutes.

Vitreous hemorrhage was identified when either macroscopic blood was observed in the vitreous or hemoglobin was detected within the vitreous fluid. For this purpose, in patients without macroscopic blood in the vitreous, hemoglobin levels were measured in the vitreous fluid by spectrophotometry (Uvikon 860; Kontron Instruments, Zürich, Switzerland) according to the classic method of Harboe¹³ for measuring plasma hemoglobin in micromolar concentration. This method has recently been validated,¹⁴ and in our hands the lower limit of detection was 0.03 mg/mL. Only the patients in whom hemoglo-

bin level was below the detection limit were considered free of intravitreous bleeding.

The protocol for sample collection was approved by the hospital ethics committee, and informed consent was obtained from patients.

ANALYSIS OF SURFACE MARKERS

Reagents

Monoclonal antibodies (mAbs) against CD3, CD28, CD8, and CD4 conjugated to fluorescein isothiocyanate, phycoerythrin, peridium chlorophyll protein, and allophycocyanin, as well as isotype IgG control mAb, were obtained (Becton, Dickinson and Co Immunocytochemistry Systems, San Jose, Calif).

Whole Blood

Cells were analyzed immediately after blood collection by means of multiparameter flow cytometry according to standard protocols described by the manufacturer. Briefly, 4-color staining of surface markers was performed by incubation of the whole blood with saturating amounts of the different mAbs mentioned previously. Stained cells were then washed with staining buffer (phosphate-buffered saline plus 1% fetal calf serum plus 0.1% sodium azide), and red blood cells were lysed with an automated sample preparation system (FACS Lyse; Becton, Dickinson and Co Immunocytochemistry Systems). After washing, cells were resuspended and then analyzed with a double laser flow cytometer (FACS Calibur; Becton, Dickinson and Co Immunocytochemistry Systems). All samples were protected from light during incubation throughout the procedure. Negative control isotype IgG-matched mAbs were used for each case. Cell analysis was performed with an acquisition and analysis program (CellQuest; Becton, Dickinson and Co Immunocytochemistry Systems).

Vitreous Fluid

Vitreous specimens were centrifuged at 2500 rpm for 15 minutes at 4°C and the pellet obtained was resuspended in staining buffer (phosphate-buffered saline plus 1% fetal calf serum plus 0.1% sodium azide). Surface markers were stained by incubation with saturating amounts of the different mAbs. Stained cells were then washed with staining buffer, resuspended, and analyzed with the flow cytometer. All samples were protected from light during incubation throughout the procedure. Negative control isotype IgG-matched mAbs were used for each case. Cell analysis was performed with an acquisition and analysis program (CellQuest; Becton, Dickinson and Co Immunocytochemistry Systems). In case of samples with vitreous hemorrhage, red cells were lysed as described earlier. Results were expressed as percentages of positive cells.

STATISTICAL ANALYSIS

Statistical analysis was performed with a microcomputer version of SPSS (SPSS Inc, Chicago, Ill). For comparisons between peripheral blood and vitreous fluid in the same patient, the Wilcoxon test was used. Levels of statistical significance were set at $P < .05$. Data are presented as median and range.

RESULTS

Three diabetic patients were excluded at the time of analysis because of the presence of nonviable cells in the vitreous fluid. Therefore, data from 17 diabetic patients were considered suitable for analysis. Six (35%) of the 17 pa-

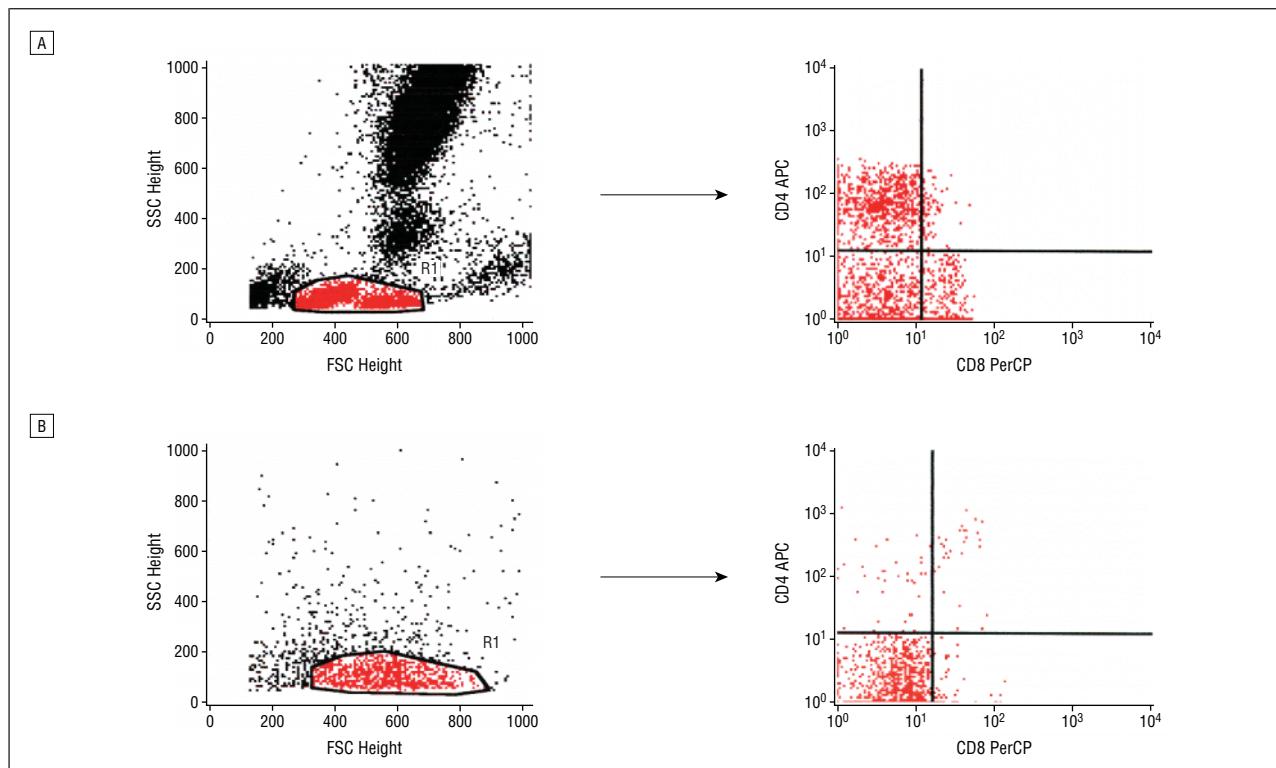


Figure 1. Whole blood (A) and vitreous fluid (B) CD4⁺ and CD8⁺ T lymphocytes from a representative patient with proliferative diabetic retinopathy without vitreous hemorrhage. Samples were stained immediately after collection with CD3-fluorescein isothiocyanate, CD4-allophycocyanin, and CD8-peridium chlorophyll protein (PerCP). First, a gate was drawn around the lymphocyte population as shown. From the gated lymphocytes, we selected CD3⁺ (T cells) and analyzed the distribution of CD4⁺ and CD8⁺ cells. SSC indicates side scatter; FSC, forward scatter.

Percentages of CD4⁺ and CD8⁺ T Lymphocytes and Expression of CD28⁺ in Peripheral Blood and Vitreous Fluid From Patients With Proliferative Diabetic Retinopathy With or Without Vitreous Hemorrhage at the Time of Sample Collection*

	Proliferative Diabetic Retinopathy			
	Nonvitreous Hemorrhage		Vitreous Hemorrhage	
	Blood	Vitreous Fluid	Blood	Vitreous Fluid
CD4 ⁺ T cells (total)	42.32 (25.49-46.25)	2.70† (0.36-12.12)	40.60 (35.03-64.92)	48.71 (0.73-63.24)
CD8 ⁺ T cells (total)	27.02 (17.42-52.47)	2.71† (1.10-9.10)	31.75 (12.49-37.84)	23.21 (0.49-34.46)
CD4 ⁺ T cells	55.15 (36.42-72.64)	50.59 (21.05-57.11)	63.17 (50.60-78.75)	62.68 (52.06-75.12)
CD8 ⁺ T cells	44.85 (27.36-63.57)	49.40 (42.88-78.94)	36.82 (21.24-49.39)	37.31 (24.87-47.93)
CD4 ⁺ CD28 ⁻	8.45 (2.43-56.59)	33.34‡ (20.75-100.00)	20.64 (10.34-41.25)	40.99 (24.32-100.00)
CD8 ⁺ CD28 ⁺	44.16 (18.04-66.31)	67.32 (0.00-100.00)	19.85 (17.73-68.93)	21.05 (0.00-45.21)

*Data are median (range percentages). The first 2 rows represent the percentages of CD4⁺ and CD8⁺ T cells detected among the total viable cells observed, while the rest of the rows represent the percentage of T-cell subsets among the total number of T lymphocytes. This relative percentage has been used for statistical analysis.

† $P < .001$.

‡ $P = .02$.

tients with PDR and none of nondiabetic controls had vitreous hemorrhage. For the purpose of the study, data from diabetic patients with vitreous hemorrhage were analyzed separately.

T lymphocytes were detected in all diabetic patients with vitreous hemorrhage, in 6 (55%) of 11 diabetic patients without blood in the vitreous, and in none of the nondiabetic patients. A flow cytometric analysis of both blood and vitreous fluid from a representative patient with PDR is displayed in **Figure 1**. The percentages of T cells obtained, as well as their phenotypical analysis in periph-

eral blood and in the vitreous fluid according to the presence or absence of vitreous hemorrhage, are presented in the **Table**. As expected, the percentages of T cells, as well as the percentages of CD4⁺ and CD8⁺ in the vitreous fluid and in the peripheral blood, were similar in patients with vitreous hemorrhage. In these patients, the percentage of CD4⁺ CD28⁻ T cells was higher in the vitreous fluid than in the peripheral blood, but the differences were not of statistical significance (40.99% [24.32%-100.00%] vs 20.64% [10.34%-41.25%]). In addition, we did not observe any statistical difference in the percentage of CD8⁺ CD28⁺ T

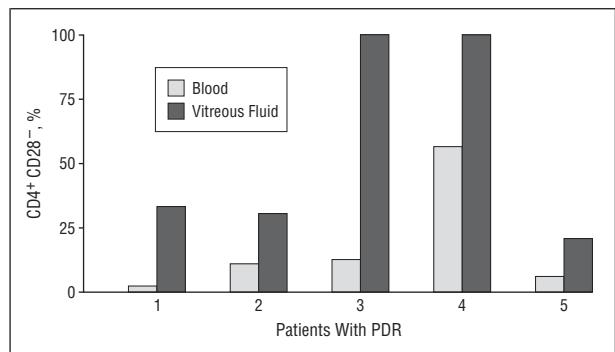


Figure 2. Percentage of CD4⁺ CD28⁻ T lymphocytes in the vitreous fluid and peripheral blood in patients with proliferative diabetic retinopathy (PDR) without vitreous hemorrhage (n=6). In one case this percentage could not be calculated for technical reasons. The percentage of CD4⁺ CD28⁻ was higher in the vitreous fluid than in peripheral blood in all patients in whom it was analyzed (n=5).

cells between the vitreous fluid and the peripheral blood (21.05% [0.00%-45.21%] vs 19.85% [17.73%-68.93%]).

Patients without vitreous hemorrhage showed a slight, nonsignificant reduction in the percentage of CD4⁺ and a mild increase in the percentage of CD8⁺ in the vitreous fluid in comparison with the peripheral blood (50.59% [21.05%-57.11%] vs 55.15% [36.42%-72.64%], and 49.40% [42.88%-78.94%] vs 44.85% [27.36%-63.57%], respectively). However, the percentage of CD4⁺ CD28⁻ T cells in the vitreous fluid was significantly higher than in the peripheral blood (33.34% [20.75%-100.00%] vs 8.45% [2.43%-56.59%]; $P=.02$), and this difference was observed in every patient (Figure 2). By contrast, CD28 was present in a greater proportion of CD8⁺ T lymphocytes in the vitreous fluid than the peripheral blood, although in this case the differences were not statistically significant (67.32% [0.00%-100.00%] vs 44.16% [18.04%-66.31%]).

The relationship between the flow cytometric analysis and both the activity of retinopathy and clinical outcome is displayed in Figure 3. All patients with PDR and no vitreous hemorrhage who showed T lymphocytes in the vitreous fluid had quiescent retinopathy, and only 1 of these patients developed early intravitreal bleeding. The remaining diabetic patients continued free of rebleeding after a follow-up of 8±2 months. By contrast, 3 of 5 diabetic patients without T lymphocytes in the vitreous fluid had early intravitreal hemorrhage.

COMMENT

Analysis of the vitreous fluid obtained from diabetic patients subjected to vitreoretinal surgery is a useful means of indirectly exploring the events that are taking place in the retina. However, there is growing evidence that the vitreous participates actively in the etiopathogenesis of PDR by means of accumulating angiogenic factors such as vascular endothelial growth factor or by losing angiogenic inhibitors such as pigment epithelium-derived factor.¹⁵⁻¹⁷ In addition, an enhancement of intravitreous concentrations of several cytokines has been reported,¹⁸⁻²⁶ thus underlining the importance of the inflammatory process in the development of PDR. In the

present study, we investigated the presence of T-lymphocyte subsets in the vitreous fluid and compared the results with those obtained in the peripheral blood at the time of vitreoretinal surgery. One of the major problems in any technique for studying these vitreous cells is to obtain an adequate number for analysis. Many cells in the vitreous fluid are already nonviable, and the remainder can disintegrate very quickly after collection of the sample. Immunocytochemical techniques have been used to study immune cells infiltrating epiretinal membranes in patients with PDR,^{18,22} but, to our knowledge, flow cytometry has not been previously applied to immunophenotyping T cells in the vitreous fluid of patients with PDR. The experimental approach used (vitreous fluid and whole-blood immunofluorescence, performed immediately after collection) allows us to simulate the *in vivo* scenario as closely as possible. The second problem when vitreous fluid is analyzed is to exclude vitreous hemorrhage. To circumvent this problem, the hemoglobin levels within the vitreous fluid were measured by spectrophotometry in all samples. Thus, only patients in whom hemoglobin was undetectable were considered free of vitreous hemorrhage.

T lymphocytes were not detected in the vitreous fluid of nondiabetic patients. This finding supports the concept that the disruption of the blood-retinal barrier is crucial for permitting the access of inflammatory cells into the vitreous body. By contrast, T lymphocytes were detected in all patients with blood in the vitreous, and the percentage of T cells as well as the pattern of CD4⁺ and CD8⁺ was very similar to that obtained in the peripheral blood. This was not a surprising result because intravitreal cellularity probably reflects peripheral blood entering the intraocular cavity. It must be emphasized that this event was found at micromolar concentrations of hemoglobin and demonstrates that a little bleeding is sufficient to change the pattern of T cells detected within the vitreous of diabetic patients with PDR.

In patients without detectable hemoglobin, T lymphocytes were detected in only 55% of cases, and the T-cell count was lower than that obtained in patients with blood in the vitreous. The substantial variations obtained in the percentages of T cells and their percentages within the total number of viable elements in the vitreous suggest that the participation of the immune system in the etiopathogenesis of PDR could be different in each patient. In addition, in these patients we found a slight deficit of CD4⁺ (helper-inducer) T cells and a mild increase of CD8⁺ (cytolytic-cytotoxic) T cells in relation to peripheral blood. However, the most important finding of the present study was the significant enhancement of CD4⁺ CD28⁻ detected in the vitreous fluid in comparison with peripheral blood in patients without vitreous hemorrhage. Activation of T lymphocytes requires 2 signals. The first signal, induced by the interaction of the antigen-major histocompatibility complex with the T-cell receptor, determines the antigen specificity, whereas the second costimulatory signal determines the activation threshold and the functional outcome of the antigen-specific activation.^{27,28} CD80/CD86-CD28/CTLA-4 is the most important and best-studied costimulatory pathway. CD80 and CD86 molecules are expressed on acti-

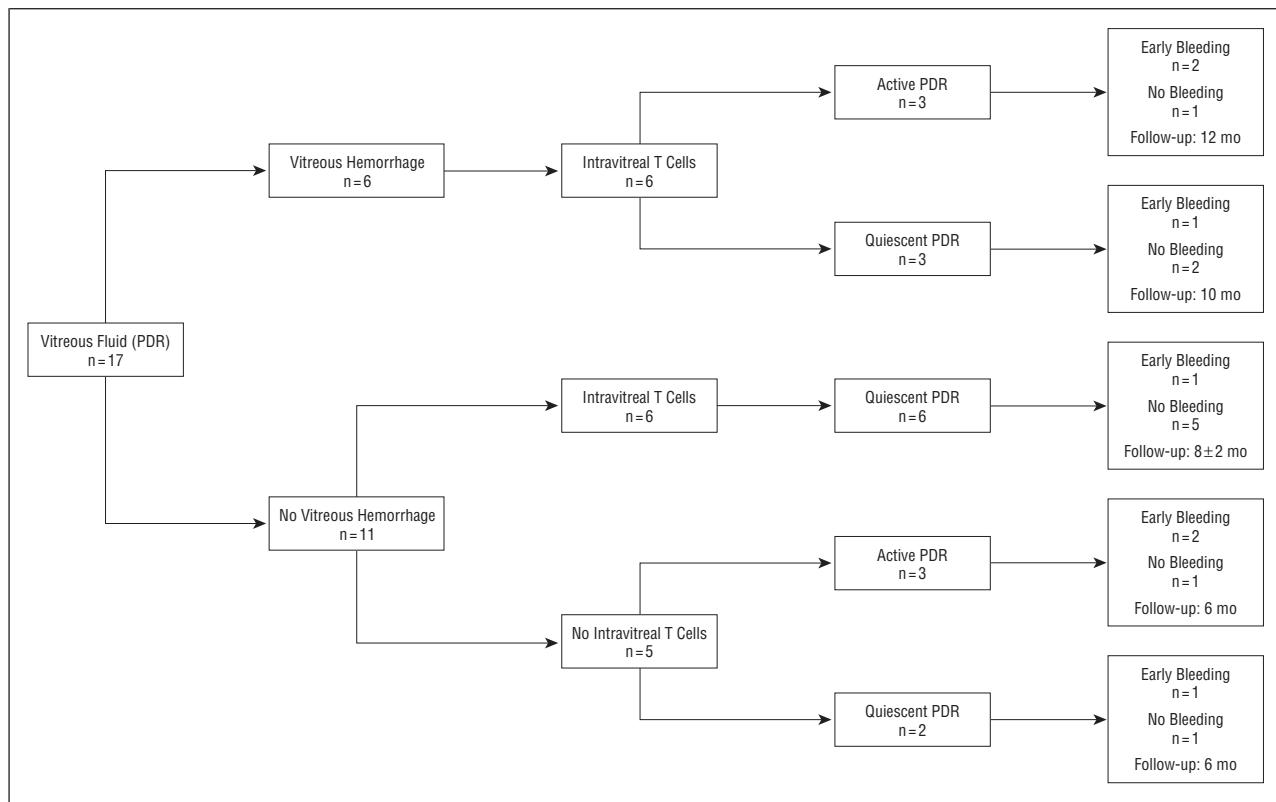


Figure 3. Relationship between the flow cytometric analysis and both the activity of retinopathy and the clinical outcome. All patients without vitreous hemorrhage had quiescent proliferative diabetic retinopathy (PDR), and only one of them developed early bleeding (vitreous hemorrhage within the 3 months after vitrectomy).

vated antigen-presenting cells and bind to their ligands CD28 and CTLA-4.²⁹⁻³¹ CD28 costimulation of human T cells increases the expression of the intrinsic cell survival factors Bcl-X_L and interleukin 2 (IL-2), which correlate with enhanced resistance to apoptosis.³² In addition, it has been demonstrated that ligation of CD28 results in the activation of protein kinase B,³³ a key mediator of growth factor-induced cell survival.³⁴ CD28 also up-regulates expression of both the IL-2 receptor and several other cytokines or chemokines (ie, IL-4, IL-3, interferon γ , tumor necrosis factor, granulocyte-macrophage colony-stimulating factor, IL-8, and RANTES [regulated on activation of normal T cells expressed and secreted]), and also modulates expression of the chemokine receptors CCR5, CCRI, CCR4, CXCR1, and CXCR2.^{35,36} Furthermore, manipulation of the CD28 pathway of costimulation can prevent the initiation of an autoimmune response, as well as suppress an ongoing autoimmune process.³⁷ For these reasons it could be speculated that, although we have detected a high percentage of CD4⁺ CD28⁻ in the vitreous fluid of diabetic patients, these T cells are lacking appropriate activation. However, the high intravitreal levels of several cytokines and inflammatory mediators detected in diabetic patients with PDR argue strongly against this hypothesis. Recently it has been demonstrated that CD4⁺ CD28⁻ clones are CD28-costimulatory independent and exhibit a full agonist signaling activation pattern, prominent T_H1 cytokine production, and a prolonged response in vitro.³⁸ Long-term memory CD4⁺ CD28⁻ cells produce high amounts of interferon γ and maximally up-regulate

interferon γ and IL-12R β 2 chain expression in the absence of costimulation. In addition, they have an increased survival after apoptotic stimuli, probably related to their persistent lack of CTLA-4 surface expression.³⁸⁻⁴⁰

The reason for the enhancement of CD4⁺ CD28⁻ observed in the vitreous fluid of diabetic patients is as yet unknown. One possibility is that costimulation-independent autoreactive CD4⁺ cells undergo activation in the periphery by the mechanism of molecular mimicry or bystander activation. Subsequently, activated CD4⁺ CD28⁻ cells may preferentially tend to leave the bloodstream, migrate through the impaired blood-retinal barrier, and initiate an inflammatory response within the eye. In this regard, costimulation-independent activation is particularly important for antigen recognition within the posterior chamber of the eye, where competent antigen-presenting cells are sparse. Alternatively, the resistance to the apoptosis reported for CD4⁺ with lack of CD28 surface expression³⁸⁻⁴⁰ could be a reliable explanation for its increased percentage within the vitreous fluid of diabetic patients.

We have observed a tendency toward a lower proportion of CD4⁺ CD28⁻ T lymphocytes in the vitreous fluid of diabetic patients without vitreous hemorrhage compared with peripheral blood. Similar results have been reported by Svenningsson et al⁴¹ in the cerebrospinal fluid of patients with either multiple sclerosis or central nervous system infectious disease. There are conflicting results in the literature regarding the role of CD28 expression in determining functional characteristics of CD8⁺ T

lymphocytes. Original data supporting primarily suppressor functions for the CD28⁻ populations^{42,43} have recently been challenged.⁴⁴ It is thus not possible at present to assign a lack of CD28 expression as being specific for cells with suppression function among CD8⁺ T lymphocytes, and so a reliable marker for this functional subset is still lacking.

One interesting finding observed in the present study is the relationship detected in patients without blood in the vitreous between flow cytometric analysis and both the activity of retinopathy and the outcome in terms of early bleeding after vitrectomy. Thus, all patients in whom T lymphocytes were detected in the vitreous fluid had quiescent retinopathy, and only 1 patient developed early intravitreal bleeding. Therefore, it seems that T cells infiltrating the vitreous cavity have a protective role in the outcome of PDR. In this regard, it should be emphasized that the neuroprotective effect of autoimmune T cells has recently been reported.^{45,46} In addition to anti-inflammatory cytokines like IL-10 or transforming growth factor β , neurotrophic factors could be potential candidates to explain the protective effect of T cells on PDR outcome.^{47,48}

In summary, in patients without intravitreous hemorrhage in whom T cells are detectable within the vitreous fluid, we found a high percentage of CD4⁺ CD28⁻ in comparison with peripheral blood. In addition, all of these patients had quiescent retinopathy and their outcome was better than that in either patients with blood in the vitreous or patients in whom intravitreous T cells were undetectable. The different pattern of T cells identified in the vitreous fluid of diabetic patients with PDR requires further functional characterization, which should provide us with a better understanding of events involved in the development of autoimmune response in diabetic retinopathy and would help us in the search for effective treatment for this disease.

Submitted for publication March 19, 2003; final revision received September 25, 2003; accepted October 14, 2003.

This study was supported by grants from the Ministerio de Ciencia y Tecnología (PM-99-0136), Madrid, Spain; Instituto Carlos III (G03/212 and C03/08), Madrid; and Novo Nordisk Pharma SA (01/0066), Madrid.

We thank Carlos Mateo, MD, for his generous collaboration in the collection of vitreous samples. In addition, we thank X. Montalban, MD, for his technical support and Michael Willi for his assistance with manuscript preparation.

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ARCHIVES Web Quiz Winner

Congratulations to the winner of our January quiz, Manpreet Singh Chhabra, MBBS, Dr Rajendra Prasad Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, New Delhi. The correct answer to our January challenge was primary orbital melanoma. For a complete discussion of this case, see the Clinicopathologic Reports, Case Reports, and Small Case Series section in the February ARCHIVES (Mandeville JTH, Grove AS; Dadras SS, Zembowicz AM. Primary orbital melanoma associated with an occult episcleral nevus. 2004;122:287-290).

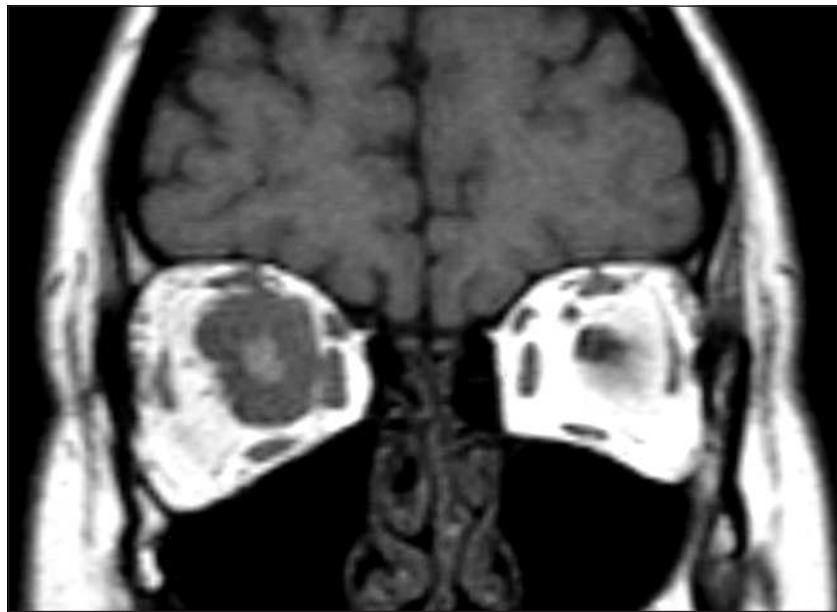


Figure 1. Magnetic resonance imaging of the orbits. Coronal, non-contrast-enhanced, T1-weighted image reveals a tumor surrounding the right optic nerve.

Be sure to visit the *Archives of Ophthalmology* Web site (<http://www.archophthalmol.com>) and try your hand at our Clinical Challenge Interactive Quiz. We invite visitors to make a diagnosis based on selected information from a case report or other feature scheduled to be published in the following month's print edition of the ARCHIVES. The first visitor to e-mail our Web editors with the correct answer will be recognized in the print journal and on our Web site and will also be able to choose one of the following books published by AMA Press: *Clinical Eye Atlas*, *Clinical Retina*, or *Users' Guides to the Medical Literature*.