Class II Histocompatibility Antigen Expression by Cellular Components of Vitreous and Subretinal Fluid in Proliferative Vitreoretinopathy

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Proliferative vitreoretinopathy (PVR) is the major cause of failure in retinal detachment surgery. It is characterized by the formation of membranes extending along both surfaces of the detached retina and within the vitreous, but the nature of the growing cells has not yet been determined. Using cytologic and immunocytologic procedures with 13 different monoclonal antibodies directed against Class II histocompatibility antigens and various markers of epithelial and immunocompetent cells, 30 specimens were studied of vitreous or subretinal fluid removed from patients with PVR. Five main types of cells could be identified: heavily pigmented cells, poorly pigmented ones, large totally unpigmented macrophage-resembling ones, smaller unpigmented cells, and lymphocytes. Analysis of intravitreal pigment granules, using autofluorescence by epillumination and cytologic procedures, showed two different populations of pigmented cells: one with autofluorescent lipofuscin granules and the other with exclusively melanin pigment. Immunostaining procedures confirmed the epithelial nonmacrophage lineage of the intravitreal and subretinal cells because most of these cells were positive for cytokeratin but negative for macrophage markers. In addition, 40–100% of these epithelial-derived cells strongly expressed Class II histocompatibility antigens HLA-DR and -DQ. Lymphocytes were found in 13 specimens; B-cells were seen, but no T-lymphocytes could be identified. These results confirm the involvement of retinal pigment epithelial cells and the strong morphologic changes they undergo during the course of PVR. Moreover, the expression of Class II histocompatibility antigens by the growing cells may be related to inflammatory phenomena, but their eventual role in the development and the extension of periretinal proliferation has not been determined. Invest Ophthalmol Vis Sci 32:2065–2072, 1991

Proliferative vitreoretinopathy (PVR) is a common complication of rhegmatogenous retinal detachment characterized by migration and proliferation of various types of cells on both surfaces of the detached retina and within the vitreous body. Continuous synthesis of collagen and contractile elements by the growing cells results in the formation of epiretinal or subretinal membranes, causing extensive retinal retraction and failure in retinal reattachment surgery. Several histologic studies have been conducted on proliferative tissues in PVR and showed macrophages, fibroblasts, pigment epithelial cells, and glial cells as components of the epiretinal membranes. Ultrastructural characteristics of fibroglial membranes are thus well documented, but only a few studies examined the cellular elements spreading throughout the vitreous body and subretinal fluid during PVR. Moreover, in previous immunohistologic studies, we found immunoglobulin (Ig) and complement deposits and abnormal Class II histocompatibility antigen expression in the pars plana and epiretinal membranes at the pigment epithelial cell level, indicating eventual involvement of the immune system during the course of PVR. We wished to characterize the cellular components of the different fluids surrounding the detached retina and proliferative tissues better and to investigate Class II histocompatibility antigen expression by intravitreal and subretinal cells in PVR.

Materials and Methods

This study was done on 30 specimens, 16 samples of vitreous and 14 of subretinal fluid, obtained surgically in 23 patients (age range, 16–78 yr) with retinal detachment and PVR as described by the Retina Society Terminology Committee. Retinal detachments were 1 week to 2 months in duration. In eight cases, unsuccessful retinal detachment surgery, including cryotherapy and scleral buckling, was done.
previously. Six patients underwent cataract surgery 4 months to 5 years before retinal detachment. In the other nine patients, no ocular surgery was done. In all cases, pigmented dots and clumps could be seen bi- 
microscopically in the vitreous, and the detached re-
tina was retracted partially or totally by fibroglial membranes spreading along the inner and/or outer surfaces of the retina.

Vitreous was removed by posterior vitrectomy through the pars plana from 16 patients. The vitreous gel was removed completely except for the peripheral vitrectomy. Subretinal fluids were aspirated from the most highly elevated retinal area, after heat coagulation of the sclera and choroid to prevent bleeding. Contaminated samples were discarded. In seven patients, both vitreous and subretinal fluid could be collected.

First all these specimens were centrifuged at 1600 rpm for 10 min. Cytocentrifuge smears were then prepared (1200 rpm, 10 min) and fixed for 10 min in methanol at 4°C. Cytologic examination was conducted using MayGrünwald Giemsa, periodic acid-Schiff, and alpha-naphthyl acetate esterase staining procedures. Toluidine blue was used to identify the nature of intracellular pigment granules; melanin granules appear brownish yellow and lipofuscin greenish-blue when stained with this dye.12 Specimens also were examined immunocytologically with 13 different monoclonal or polyclonal antibodies, directed against histocompatibility antigens HLA-DR (OKDR and I2; Ortho and Coulter, respectively), HLA-DQ (IOT2d; Immunotech), epithelial markers, epithelial membrane antigen (EMA; Dakopatts), 56-kilodalton cytokeratin (clone KL1; Immunotech), glial fibrillary acidic protein (GFAP; Dakopatts), fibronecctin (Immunotech), macrophage (OKM5; Ortho, and M02; Coulter), and lymphocyte markers (T3, T4, T8, and B4; Coulter). Antilymphocyte markers were tested only when standard cytologic examination showed the presence of lymphocytes or lymphocyte-resembling cells.

Immunofluorescence and immunoperoxidase procedures were done with previously described methods.9 Briefly, the primary antibodies in a 1:50 dilution were layered on slides for 1 hr, before washing in phosphate-buffered saline at pH 7.4, and incubation for 30 min with the secondary antibody, fluorescein isothiocyanate-labeled anti-rabbit (for anti-GFAP antisemur) or anti-mouse IgG antisemur (Dakopatts). For immunoperoxidase procedures, biotin-conjugated anti-mouse IgG antisemur was used as secondary antibody. Then avidin-biotin peroxidase complexes (Dakopatts) were reacted for 30 min before developing in aminoethyl carbazole hydrogen peroxide solution. The percentages of positive cells were obtained by counting at least 100 cells in each specimen. Damaged cells or cells without nuclei were not counted.

Negative control reactions were prepared by omission of the primary antibody or substitution by serum from the same species. In each patient, positive controls for markers of immunocompetent cells were ob-
tained by the same technique on peripheral blood lymphocytes. To assess the nature of pigment gran-
ules in ciliary and retinal pigment epithelial cells, 5-
μm thick whole-eye frozen sections were prepared from six eyes obtained by autopsy in six subjects with-
out any known ocular disease. Autofluorescence analy-
ysis and toluidine blue staining were done on these sections.

**Results**

The results of cytologic examination of vitreous specimens was independent of the length or impor-
tance of the retinal detachment and proliferative pro-
cesses. There was no difference between cellular pat-
terns from patients who previously underwent cata-
rect or retinal detachment surgery and those without any previous surgical intervention.

Five major cellular components were found as fol-
lows.

1. Rounded typical pigment epithelial cells (Fig. 1), with a large heavily pigmented cytoplasm masking the nucleus (5–30% of total vitreous cells), were seen in 13 specimens. Under epillumination, two different kinds of pigmented cells were found: 30–70% contained yellow autofluorescent pigment granules, identified as lipofuscin by toluidine blue staining. In contrast, the other heavily pigmented cells did not contain lipofuscin but only nonauto-
fluorescent pigment, identified as melanin by tolui-
dine blue.

2. Large partially pigmented cells (30–50%), with rare granules of melanin and/or lipofuscin and ap-
parently empty vacuoles, were found (Fig. 1). Nu-
clear anomalies often were seen, such as notches, duplication, and even triplication.

3. Large rounded totally unpigmented cells (Fig. 1), with similar nuclear abnormalities and intracyto-
plasmic vacuoles, were found (10–30%). Although
Fig. 1. Cytologic staining of intravitreal cells collected in a case of proliferative vitreoretinopathy, showing a heavily pigmented cell (arrowhead) and four macrophage-resembling cells containing various amounts of pigmented dots (arrows). These different kinds of cells were identified by immunocytologic procedures as epithelial-derived cells (May Grünwald Giemsa, original magnification ×550).

resembling macrophages, these cells were negative to specific macrophage staining with alphアナphantyl acetate esterase.

4. Smaller unpigmented cells (10–50%), with a condensed rounded or lightly elongated cytoplasm surrounding a large nucleus, were seen (Fig. 2). In some specimens, occasional spindle-shaped cells, with morphologic features of fibroblasts, were found.

5. Rare well-characterized lymphocytes were seen in six vitreous specimens. The absence of red blood cells confirmed the lack of hematogenic contamination of the specimens.

Identification of these different kinds of cells was achieved with immunocytologic procedures (Table 1). In 40–80% of intravitreal cells, either heavily pigmented or totally unpigmented ones, large or smaller, were positively stained for EMA and cytokeratin (Fig. 2), confirming their epithelial origin. There was no reaction to antimacrophage monoclonal antibodies. Cells with different morphologies had a similar pattern of reactivity and comparable percentages of positivity to cytokeratin. Fibronectin was found in four specimens on a minority of intravitreal cells (5–30%), mainly small unpigmented ones. In contrast, GFAP was negative. The fifth type of cells, lymphocytes, appeared to be mostly B-cells (B4 positive), with T3, T4, and T8 monoclonal antibodies negative in all specimens.

Moreover, immunofluorescence and immunoperoxidase procedures on vitreous fluid showed numerous Class II histocompatibility antigen-expressing cells (Figs. 3, 4). A strong reactivity was found in 40–100% of intravitreal cells. Both pigmented and non-pigmented, large and smaller cells widely expressed HLA-DR and -DQ determinants (Table 1). There was no difference in these percentages or the pattern reac-

Fig. 2. Immunofluorescence reactivity of anti-cytokeratin monoclonal antibodies in a small, partially elongated intravitreal cell. Nucleus is counterstained with propidium iodide (×800).
Table 1. Immunostaining reactivity of cellular components of vitreous gel (V) and subretinal fluid (SRF) in proliferative vitreoretinopathy

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Mean percentages of positive cells (SD)</th>
<th>Range (%)</th>
<th>No. of positive specimens</th>
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<tbody>
<tr>
<td></td>
<td>V</td>
<td>SRF</td>
<td>V</td>
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<tr>
<td>Cytokeratin</td>
<td>74 (18.3)</td>
<td>68 (14.1)</td>
<td>50-80</td>
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<tr>
<td>EMA*</td>
<td>58 (15.7)</td>
<td>66 (17.3)</td>
<td>40-75</td>
</tr>
<tr>
<td>anti-HLA DR</td>
<td>67 (28.9)</td>
<td>82 (20.6)</td>
<td>40-100</td>
</tr>
<tr>
<td>anti-HLA DQ</td>
<td>59 (23.6)</td>
<td>76 (18.2)</td>
<td>40-90</td>
</tr>
<tr>
<td>GFAP</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>6 (5.3)</td>
<td>—</td>
<td>0-30</td>
</tr>
<tr>
<td>OKM5 and M02</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>T-cell markers†</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>B-cell markers†</td>
<td>2 (1.5)</td>
<td>3 (2.7)</td>
<td>0-5</td>
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* Not performed in six specimens.
† Only performed when lymphocytes could be seen on cytologic examination.

Activity between the different kinds of cells and between melanin- or lipofuscin-containing cells. There was no relationship between the expression of Class II antigens and the importance of pigmentation. Both intraocular cells from patients with and without previous ocular surgery similarly expressed these antigens.

With subretinal fluid analysis, similar results were found. Cytologic examination also showed heavily pigmented, lightly pigmented, and the two different types, small and large, of unpigmented cells. In seven specimens, a few lymphocytes could also be seen. However, in contrast with vitreous samples, autofluorescent lipofuscin granules could be observed under epillumination in all pigment-containing cells. Subretinal cells (Fig. 5) also strongly expressed HLA-DR and -DQ determinants on their membranes. There was no difference in the percentages of positivity between the four kinds of cells, pigmented or unpigmented, large or small. Both pigmented and unpigmented cells widely reacted with EMA and cytokeratin. No cell was positive for antimacrophage antibodies, and macrophage-specific staining was negative on all cells. A few B-lymphocytes were found in seven specimens, but the other tested antibodies (fibronectin, GFAP, T3, T4, and T8) were negative.

Pigment examination on normal eye sections showed, either by autofluorescence or toluidine blue staining, a sharp delineation between retinal pigment epithelium (continuously and strongly autofluorescent [Fig. 6A] or greenish-blue when stained by toluidine blue).
Fig. 4. HLA DR antigens in two partially pigmented intravitreal cells. In contrast to Figure 3, the intracytoplasmic pigment is nonautofluorescent melanin (arrows). Note also the duplication of the nuclei (immunofluorescence, propidium iodide counterstain, original magnification ×550).

Fig. 5. Various types of subretinal cells strongly expressing HLA DQ antigens: large lipofuscin-containing (arrow) and smaller unpigmented cells can be seen which similarly express class II antigens (immunofluorescence, propidium iodide counterstain, original magnification ×550).

Several reports show that epiretinal membranes result from proliferation of various types of cells, including pigment epithelial ones (probably migrating through retinal tears) and glial cells growing through disruptions of the inner retinal membrane. Epiretinal membranes also were found to be composed of fibroblast-like and macrophage-like cells, but the origin of these cells is controversial, even though immunohistologic studies suggest that they could originate from metaplastic retinal pigment epithelial cells. Animal models of PVR show that retinal pigment epithelial cells rapidly change their morphology when implanted into the vitreous body or when they

Fig. 6. Toluidine blue and ciliary or iris pigment epithelial cells totally devoid of lipofuscin. The latter were not autofluorescent (Fig. 6B) and were stained brownish by toluidine blue. Only a few granules of lipofuscin could be seen in pigment epithelial cells of pars plana adjacent to the peripheral retina.
proliferate on the retinal surface, lose their pigment granules, and no longer resemble typical pigment epithelial cells. This may result in confusion in identification of the cell types involved in epiretinal membranes.

Fibroglial membrane formation along the retina, however, appears as the result of complex proliferative phenomena which occur very early in the course of PVR and involve other structures besides the retina. The vitreous gel of patients with PVR usually contains brown pigmented dots and clumps, even when epiretinal membranes cannot be seen, which indicates strong extraretinal cellular processes before epiretinal membrane formation. Moreover, because retinal pigment epithelial cells are supposed to proliferate actively and participate in sub- and epiretinal membrane development, studies on the subretinal fluid that accumulates in the space between the neural retina and pigment epithelium may be important in understanding the pathophysiologic events leading to the formation of this periretinal tissue. Biochemical analysis of subretinal fluid has been well documented, but cytologic studies on cellular components of subretinal fluid and vitreous gel are few.

An ultrastructural work of Feeney and associates showed two major cell types in subretinal fluid: typical pigment epithelial cells and macrophage-like pigment-containing ones. In addition to classic cytologic procedures, we used immunocytochemical methods to identify cellular components present in the vitreous and subretinal fluid of patients with PVR. We therefore found different cellular patterns such as typical pigment epithelial cells and lightly pigmented or totally unpigmented cells, including macrophage-resembling ones. Immunostaining for epithelial and macrophage markers showed evidence that these cells were of epithelial origin and not macrophage derived. They widely expressed epithelial markers, mainly cytokeratin, a characteristic determinant of retinal pigment epithelial cells. In addition, some of the observed cells, the small unpigmented ones, although morphologically different from typical pigment epithelial cells, were also identified as epithelial-derived cells. In contrast was the absence of GFAP-positive glial cells, even though they have been found in epiretinal membranes. We suggest that glial cells probably spread along the detached retina but do not reach the vitreous cavity or subretinal space. A minority of cells in each different morphologic pattern, however, did not react with our markers and could not be precisely identified. In all cases, these cells were negative to all the tested antibodies, and the absence of any kind of reactivity could be related to cellular alterations during specimen collection.

These patterns could thus be the result of strong ultrastructural changes occurring in proliferating pigment epithelial cells. They are consistent with those of animal models of PVR, in which cultured retinal pigment epithelial cells are injected into the vitreous body. Similarly, retinal pigment epithelial cells overlaid with vitreous, collagen, or fibrin have been shown to lose their normal epithelial characteristics and, gaining fibroblast-like features, migrate into the surrounding extracellular matrix. Vitreous stimulation of pigment epithelial cells through retinal tears may thus play a role in the development of PVR.

Moreover, characterization of lipofuscin and melanin content, either by autofluorescence evaluation or histochemical staining, showed that about 50% of the intravitreal strongly pigmented cells contained only melanin and not lipofuscin granules. The large amounts of pigment granules in these cells could not result from phagocytosis of intravitreal debris. Lipofuscin pigments result from a deficient degradation of
phagocytized shed outer segments in intracytoplasmic phagolysosomes. They begin to accumulate in retinal pigment epithelial cells as early as the 16th postnatal month. The presence of intravitreal cells containing large amounts of melanin pigment without any lipofuscin granules suggests that retinal pigment epithelial cells are not the only ones involved in proliferative phenomena during PVR and that ciliary process or iris pigment epithelial cells, which are devoid of lipofuscin pigment as assessed on normal eye sections, could also migrate and proliferate in the vitreous body. This is consistent with our previous immunohistologic study done on pars plana specimens from patients with PVR. We showed the involvement of ciliary pigment epithelial cells, on which Ig and complement deposits and abnormal expression of Class II major histocompatibility complex antigens could be seen. These antigens also were found at the surface of numerous pigmented cells in epiretinal membranes in PVR. In the current study, most intravitreal and subretinal cells, whether heavily or lightly pigmented or unpigmented, strongly expressed HLA-DR and -DQ antigens.

Class II antigens are membrane glycoproteins directly involved in the presentation of an antigen to helper T lymphocytes. They are normally restricted to immunocompetent cells, such as macrophages, B lymphocytes, or activated T cells, but aberrant expression of Class II antigens by resident epithelial cells has been documented in various autoimmune diseases. The hypothesis that expression of Class II antigens by target cells might enable them to present antigens to immunocompetent cells such as helper T lymphocytes which could initiate an autoimmune reaction was proposed by Bottazzo and associates. In the eye, HLA-DR was expressed by retinal pigment epithelial cells in retinitis pigmentosa, uveitis, and PVR. Moreover, experimental studies stressed the prominent role of Class II expression in the initiation and perpetuation of intraocular immune responses. Class II antigen expression by retinal pigment epithelial cells is one of the earliest events in the development of experimental autoimmune uveoretinitis and precedes induction of local inflammation. In addition, immunotherapy by monoclonal antibodies to Class II determinants has been shown to reduce the course of various experimentally induced autoimmune diseases. Anti-Class II antigen treatment was efficient in inhibiting or suppressing ocular inflammation in experimental autoimmune uveoretinitis, even when the antibodies were administered 5–7 days after antigen injection.

Our studies indicate that pigment epithelial cells during the course of PVR express HLA-DR and -DQ determinants in the ciliary body, epiretinal membranes, vitreous gel, and subretinal fluid. Normal ciliary and retinal pigment epithelial cells do not. However Class II antigen expression by retinal pigment epithelial cells in PVR is not the consequence of inflammation induced by previous surgical interventions. Nine of our 23 patients had not undergone any ocular surgery before specimen collection.

Even if Class II antigen induction on proliferative cells is only an epiphenomenon resulting from a nonspecific inflammatory reaction, we can hypothesize that the expression of HLA-DR and -DQ antigens by retinal pigment epithelial cells or pigment epithelial-derived ones during the development of PVR may allow them to function as effective antigen-presenting cells and induce an immune reaction. Some reports cite the possibility of autoimmune phenomena in retinal detachment. Using assays of cellular immunity, patients with long-standing retinal detachment or epiretinal proliferation showed autoimmune responses to retinal or uveal antigens. Serum antibodies to retina also were detected in 60% of patients with retinal detachment. More recently, experimental studies in animal models of retinal detachment showed cellular immunity to three purified retinal antigens: interphotoreceptor retinoid-binding protein, S-antigen, and opsin. Our findings of lymphocytes in vitreous and subretinal fluid, together with previous reports on the presence of lymphoid cells in experimentally induced epiretinal membranes, provide additional indications on the intervention of the immune system in PVR.

Mechanisms of such immune involvement are speculative, but Class II antigen expression by the proliferating retinal pigment epithelial cells and the eventual subsequent immune response could be dependent on the liberation of some of the growth factors which lead to periretinal cell proliferation after retinal detachment. Various growth factors, known to stimulate proliferation of retinal pigment epithelial cells, such as platelet-derived growth factor, epidermal growth factor, and fibroblast growth factor, enhance the production of gamma interferon, a potent inducer of HLA-DR expression by retinal pigment epithelial cells both in vitro and in animal models. Some of these growth promoting factors, possibly liberated from their storage sites after retinal detachment, may induce the target cells to proliferate and, at the same time, to express Class II antigens, resulting in additional inflammatory phenomena.

However it remains to be determined whether or not the Class II antigen-mediated immune phenomena play a role in the development of PVR and the extension of epiretinal membranes after retinal detachment. Further studies are needed to identify the respective roles of growth factors, class II antigen expression, and immune mediators in proliferative diseases.
Key words: Class II antigens, retinal detachment, proliferative vitreoretinopathy, vitreous, retinal pigment epithelium

References