Ultrastructural Pathology of S-Antigen Uveoretinitis

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The morphology of S-antigen–induced uveoretinitis in guinea pigs has been studied using transmission electron microscopy. Purified bovine retinal S-antigen was shown to produce a focal chorioretinitis, characterized by selective damage to the outer retina and, almost exclusively, a mononuclear cell infiltration of the choroid and retina. Even at high doses, extensive rod outer segment damage was associated predominantly with lymphocytic and mononuclear cell infiltration. A single immunizing injection of S-antigen was sufficient to produce a chronic ocular inflammation lasting many months. Focal lesions evolved rapidly and reached an end-stage within days to weeks. Accordingly, eyes examined at any time during the disease contained areas of normal retina coexistent with fibrotic lesions. With time, the number of advanced or end stage lesions became more frequent, thereby involving a more widespread area of the retina. Examination of early stage lesions suggest that the rod outer segment is the target for immune damage in this disease, but the mechanism of damage remains to be elucidated. Invest Ophthalmol Vis Sci 26:1281–1292, 1985

Experimental allergic uveoretinitis (EAU) has been used widely as a model for autoimmune retinal disease. The disease can be induced by a variety of extracts of retinal tissue in complete Freund’s adjuvant injected at distant sites, including whole retina, crude soluble retinal extract, crude rod outer segment preparations and rhodopsin. The most potent immuno-pathogenic agent in autoimmune retinal disease is a soluble antigen (S-antigen) from retina1,2 since it is effective in microgram doses while other antigens such as rhodopsin usually only have effect at milligram doses and higher.3,4

Rao et al5 reported a systematic light microscopic study of the effects of purified S-antigen in the guinea pig. High doses of S-antigen induced severe panuveitis while low doses produced a much less severe form of the disease. Marak et al6 showed that the high dose response could be inhibited by complement depletion. Faure et al7 also induced extensive uveoretinitis with 100- and 50-μg doses of S-antigen in monkeys, but in this case it was predominantly a posterior uveitis. However, in rats, a severe anterior and posterior uveitis was induced,8 and histologically a retinal vasculitis with polymorphonuclear cellular infiltration was observed.

The present study documents the ultrastructural changes in S-antigen–induced uveoretinitis in guinea pigs. Our purpose was to investigate the evolution of the disease at varying doses and to identify the target organ, if any, and its mode of destruction. Highly purified S-antigen prepared by a modified technique9 and shown to have immunological identity with S-antigen prepared by Wacker et al1 was used to produce EAU in guinea pigs. At all doses up to 135 μg per animal, a posterior uveitis only was observed and this was predominantly a focal mononuclear cell reaction with little evidence of a polymorphonuclear leukocyte response. Low doses of S-antigen appeared to produce preferential photoreceptor damage associated with macrophages or round cells in the subretinal space. These findings support the view that the rod outer segment (ROS) is the target organ for damage in EAU.

Materials and Methods

Animals

Duncan-Hartley and Peruvian Abyssinian pigmented guinea pigs (200–300 g) of both sexes were used for the induction of EAU. Methods used conformed to the ARVO Resolution on the Use of Animals in Research.

Preparation of Retinal S-antigen

S-antigen was extracted from bovine retinae as described previously.9 Briefly, bovine retinae were homogenised in cold hypotonic (0.0025 M) Tris HCl buffer, pH 7.9, centrifuged at 80,000 × g for 1 hr and the supernatant precipitated with ammonium
Table 1. Number of animals at each time point

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<th>Antigen dose (μg)</th>
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<th>Late phase (wk)</th>
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<td>Total</td>
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* Figures in parentheses indicate number of animals of strain ABX. All other animals were Duncan-Hartley strain.
† Two of these animals were immunized with S-antigen prepared on a chromatofocusing column.

Immunization Schedule

Animals received a single footpad injection of S-antigen in Freund’s complete adjuvant (H37 Ra, Difco; Detroit, MI). The antigen and adjuvant were mixed in equal proportions of 0.1 ml. Various doses of immunizing antigen were used (17, 35, 100, 135 μg total dose per animal) and controls included saline/adjuvant alone. Animals were killed at various time intervals after immunization. The number of animals used at each time point is indicated in Table 1. The animals have been grouped according to the dose of antigen used (low or high dose) and timing of clinical response (early phase, late phase) (Table 1).

Clinical Assessment

The pupils of both eyes were dilated with 0.5% cyclopentolate. Clinical signs of uveoretinitis were monitored by direct slit-lamp biomicroscopy of the anterior vitreous through the dilated pupil. Active uveitis was scored in vivo according to the degree of cellular infiltration of the anterior vitreous by the method of de Kozak et al 10 on a scale of 0–4 (Table 2). Anterior segment changes including corneal haze, anterior chamber cells and flare, and iris vessel dilatation were also monitored.

Tissue Preparation for Light Microscopy and Transmission Electron Microscopy

The eyes were fixed either by rapid immersion in or by in vivo perfusion of 2–4% glutaraldehyde (cacodylate buffered pH 7.6) at 4°C. Immersion fixation was performed immediately upon death by enucleation of the eyes and 180 deg incision of the globe at the pars plana to facilitate penetration of the fixative. Perfusion fixation was performed on deeply anesthetized (ether and Hypnorm, Janssen; Kent, U.K.) animals via the aorta through an abdominal
incision. The eyes were then rapidly enucleated and placed in 2–4% glutaraldehyde.

After at least 24 hr in the primary fixative the eyes were dissected as follows. A circular incision was made immediately posterior to the limbus, dividing the eye into anterior and posterior halves. The lens was removed from the anterior half, which was then divided in several meridional planes to produce blocks which contained cornea, sclera, ciliary body, and iris. The vitreous which remained in the eye cup was examined with the aid of a dissecting microscope (×20) and the gel with visible inflammatory cell aggregates was dissected. The rest of the eye cup was also examined for evidence of focal chorioretinal lesions which were more easily detected in pigmented animals. The eye cup was divided into quadrants and then into 10–20 blocks which contained retina, chorioid and sclera. Tissue blocks (anterior segment, vitreous, and retina) were postfixed in 1% osmium tetroxide, dehydrated and embedded in Araldite in the conventional manner. Semithin sections (1–2 μm) were cut and stained with uranyl acetate and lead citrate before examination in a Philips (Phillips; London, U.K.) 301 transmission electron microscope.

Results

Clinical Assessment

A single injection of bovine retinal S-antigen produced a long-lived inflammatory cell infiltration of the vitreous as seen by slit-lamp biomicroscopy (Fig. 1). In general, higher doses produced a more severe posterior uveitic response than lower doses. All animals receiving S-antigen injections developed EAU. The most severe reaction was an intense white deposit which filled the vitreous and obliterated the normal red reflex. An anterior uveitic reaction was rarely observed and consisted of iris vessel dilatation accompanying the most severe forms of posterior uveitis. Cells and flare in the anterior chamber were not seen.

Normal Morphology

The morphology of the normal guinea pig retina had not been specifically reported although Kuwabara states that the retinal pigment epithelium (RPE) is similar to the rabbit RPE. In the present study, satisfactory preservation of the tissue was obtained with both rapid immersion fixation and perfusion fixation and most of the eyes were therefore fixed by the former method. Guinea pig retina is nonvascularized (Fig. 2a) and the outer segments are predominantly rods.

Prodromal Phase

After footpad injection of S-antigen, but before the onset of clinical uveoretinitis (less than 10 days), little change was seen in the retinal architecture. Some eyes showed occasional degeneration of the cone photoreceptor outer segments, but no inflammatory cells were seen in the retina. Occasional mononuclear cells were seen in the vitreous gel.

Active Phase

Clinical active uveoretinitis was characterized histologically by a focal chorioretinal inflammatory lesion which progressively increased in number with time. However each focal lesion had a short self-limiting progression towards an “end-stage” pathology and, as a result, a wide spectrum of morphologic appearances was seen within any one eye at any dose of S-antigen. The data reported here, therefore, represent the sequence of changes within these focal lesions that occurred after a single injection of S-antigen at low (17 and 35 μg) and high (100 and 135 μg) doses.

Low-Dose S-antigen

A single low dose (17, 35 μg) footpad injection of S-antigen produced a chronic mild uveoretinitis that
lasted several months and was characterized by multiple focal lesions. Little effect on most of the retina was observed in the early phase of the disease, i.e., within the first 2 or 3 days of clinically apparent cellular infiltration of the vitreous. In some areas of the retina, however, focal damage was evident as swelling and disruption of the photoreceptor outer segments (Fig. 3). The rod damage was accompanied by single mononuclear cells at the ROS--RPE interface. Some of these cells contained large phagocytic inclusions but in general they showed only moderate signs of activity (Fig. 3). Occasional ROS had a relatively normal disc arrangement (Fig. 3) but in the majority the rod discs had disappeared and the swollen ROS matrix was occupied by flocculent electron dense material and sparse residual membranous bodies (Fig. 3). Rod disc membranes appeared to be preferentially damaged in contrast to outer segment mitochondrial membranes which were preserved until late in the lytic process. The RPE cells showed signs of reduced phagocytic activity: loss of apical microvilli; reduced basal infoldings; few phagosomes and swelling and disruption of mitochondria. The inner segments of the photoreceptors showed considerable organelle disruption and swelling. There was also loss of cell nuclei and widespread pyknosis of the nuclear chromatin in remaining cells.

In other areas of the retina, the RPE appeared relatively normal and photoreceptor damage took the form of ROS fragmentation into discrete packets of
rod outer discs which were completely enclosed in the apical microvilli of the RPE (Fig. 4). In these areas fewer ROS had undergone swelling and lysis. Single macrophages were present in these areas but appeared distributed at all levels throughout the photoreceptor layer and not merely at the ROS-RPE interface. In addition, they contained numerous phagosomes representing ROS in various stages of digestion. With time some cells became filled with inclusions such as recently ingested ROS, amorphous phagosomes, membranous whorls, crystalline deposits and several other forms.

The inner retina and the choroid showed few changes at this stage. In particular, there was evidence of inflammatory cell invasion of the choroid only in a few focal areas. Most of these cells were mononuclear cells.

Morphologic study of eyes removed at later stages showed increasingly widespread areas of focal retinal damage (Fig. 5). In addition, damage to other retinal layers was observed such as Müller cell oedema and vacuolation of inner and outer plexiform layer. Some macrophages in the photoreceptor layer appeared to be in a state of cell lysis. End stage focal lesions, produced by low dose S-antigen showed total atrophy of the photoreceptor layer. Single macrophages within the subretinal space assumed very large proportions and were filled with large quantities of phagocytic material. The choroid contained few inflammatory cells but the choriocapillaries showed signs of capillary stasis and frequently contained leukocytes, particularly eosinophilic granulocytes.

High-Dose S-antigen

High dose of S-antigen (100, 135 μg) produced a clinically severe posterior uveitis within 2 or 3 wk. At the onset of the disease, most of the retina and choroidal architecture was normal. Some areas of the retina showed solitary macrophages and photoreceptor atrophy as seen in low dose disease. However, the most characteristic feature was a focal area of massive...
mononuclear cell infiltration into the choroid (Fig. 6). This was associated with almost total photoreceptor outer segment swelling and lysis in the absence of inflammatory cells in the subretinal space. Fragments of intact rod outer segments were identified at the interface with the RPE and the photoreceptor cell body but the rest of the ROS were in various stages of lysis with preferential loss of disc membranes to cytoplasmic membranes; hence they took on the appearance of "sacs" of flocculent debris.

The RPE showed extensive vacuolation and mitochondrial swelling, loss and/or fusion of apical microvilli, few phagosomes, and loss of basal infoldings. In addition, in some areas, cells from the choroid, presumably macrophages, had migrated between Bruch's membrane and the RPE. The choroidal inflammatory cell infiltrate was composed predominantly, of large mononuclear cells with abundant cytoplasm. Few phagosomes were noted in these cells. Occasional plasma cells were seen, as were neutrophilic and eosinophilic granulocytes.

In the chronic phase of high-dose disease, areas of normal retina became less frequent. The RPE was thinned and atrophic particularly in those areas underlying giant macrophages. Areas in which there was chronic mononuclear cell infiltration in the choroid showed variable degrees of photoreceptor atrophy with macrophage infiltration. In addition, spindle-shaped cells were seen between the RPE and Bruch's membrane (Fig. 7a). In the choroid, eosinophils became a notable feature not only for their increase in number but for their close intercellular association.

High doses of S-antigen eventually produced replacement of the photoreceptor layer with chronic

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**Fig. 4. Low-dose EAU (35 μg S-antigen, 4 wk postimmunization).** Electron micrograph showing a relatively healthy RPE with apical microvilli, enclosing rod outer segments (arrowheads). An invading cell (MC) is visible between Bruch's membrane (bm) and the RPE basal infolding. Note large macrophage, containing phagolysosomes (asterisks), apparently in the process of phagocytosing a discrete packet of ROS (arrow) amongst disrupted photoreceptor outer segments (bar = 5 μm, Duncan-Hartley strain).
Fig. 5. Low-dose EAU (35 µg S-antigen, 14 wk postimmunization). Light micrograph showing a focal chorioretinal lesion, involving the outer retina. The cellular infiltrate is predominantly mononuclear (bar = 50 µm, Duncan-Hartley strain).

Fig. 6. High-dose EAU (100 µg S-antigen, 2 wk postimmunization). Extensive infiltration of choroid with mononuclear cells and obliteration of choroidal vasculature. Complete destruction of normal ROS pattern with swelling and lysis of ROS disc membranes. Note saccular appearance of ROS fragments containing flocculent debris. Inflammatory cells are absent from ROS layer. However note cells between RPE and Bruch's (arrows) (bar = 20 µm, Duncan-Hartley strain).
Fig. 7. High-dose EAU (100 μg S-antigen, 8 wk postimmunization).
a. Photoreceptor layer is replaced by spindle-shaped cells (curved arrow). The outer nuclear layer is hypocellular and disorganized. The RPE cannot be identified. The choroid contains numerous round cells including plasma cells (arrowheads). Large choroidal arteriole (A).
b. Higher magnification of choroidal plasma cell; c, fibrillar material, probably fibrin (arrow), on upper surface of degenerate RPE. bm: Bruch's membrane (a, bar = 10 μm; b, bar = 2 μm; c, bar = 2 μm).
inflammatory cells and spindle-shaped fibroblasts (Fig. 7a). The inflammatory cell infiltrate in the choroid was also predominantly mononuclear but included plasma cells (Figs. 7a, 7b). The choriocapillaris was obliterated and only rarely was a larger choroidal vessel observed. The retinal pigment epithelium could not be identified positively in some areas. Elsewhere, electron dense material was observed at the upper surface of the degenerate RPE (Fig. 7c). At higher magnification, this material had a fibrillar matrix and showed some similarity to fibrin. In addition, some RPE cells appeared to have lost normal contact with Bruch’s membrane and were showing signs of multilayering and cell migration (Fig. 8).

The outer and inner retina showed extensive degenerative changes with loss of nuclei from the inner and outer nuclear layers.

In areas where there was minimal choroidal inflammation and less extensive ROS damage, aggregates of large macrophages were found in the subretinal space (Fig. 9). Their phagosomes were similar to those seen with low doses of antigen but they were more numerous. These cells frequently contained typical ellipsoid melanin granules from the retinal pigment epithelium.

Discussion

Light microscopic studies of experimental allergic uveoretinitis (EAU) have been reported on several occasions but most have relied upon crude retinal extracts or whole rod outer segment preparations for the induction of EAU. 12-17 More recently, pure retinal S-antigen has been used to produce EAU in guinea pigs.
pigs, rats, rabbits, and monkeys. Histologic studies of the effects of varying doses of S-antigen on the eye have been reported by Rao et al using homologous guinea pig S-antigen and in rats by De Kozak et al, also with bovine antigen. Low doses of homologous S-antigen in guinea pigs produced a mild choroidal mononuclear cell infiltration with “sparing” of the retina while high doses (up to 50 μg) produced a very severe acute necrotizing anterior and posterior uveitis with destruction of most of the retinal layers.

High doses of heterologous antigen were required to produce EAU in rats and, in most strains, the disease was mild with focal destruction of the rod outer segment layer and mononuclear cell infiltration. Only in Lewis rats was a severe form of EAU elicited and usually with high doses and double adjuvant administration.

The results of the present study differ from the above in several respects: (1) At all doses used, ie, up to 135 μg S-antigen, the disease was almost exclusively a posterior segment focal inflammation that comprised a mixed inflammatory cell infiltrate at the pars plana, diffuse cellular invasion of the vitreous, and focal cellular invasion of the choroid and outer retinal layers. The anterior segment was not significantly involved. (2) Focal chorioretinal lesions evolved rapidly such that even within a few days of onset of the disease, histologic examination of the eye revealed areas of normal retina alternating with areas of “end-stage” lesions and newly developing lesions. (3) Low doses of S-antigen produced a mild disease that was characterized by single macrophages in the photoreceptor layer, ie, there was preferential damage of rod outer segments. High doses of S-antigen produced similar photoreceptor damage, but in certain areas this was extremely acute and extensive. (4) Despite the widespread destruction of ROS at high doses of S-antigen, the cellular infiltrate remained predominantly mononuclear, although other cell types such as polymorphonuclear and eosinophilic leukocytes were occasionally seen. This contrasts with the acute necrotizing lesions with marked polymorphonuclear leukocyte infiltration reported by Rao et al in EAU with high doses of S-antigen. (5) A single immunization with S-antigen induced a long-lived chronic inflammatory disease that differs from the acute EAU models described by others.

There are several reasons that could account for the differences in morphology between this study and those reported previously. For instance, there are
significant species and strain differences in the animals used in these studies; in addition, heterologous (bovine) S-antigen was used in the present study, whereas Rao et al. used homologous antigen in guinea pigs; also, there are important differences in the method of preparation and the characteristics of the final product of S-antigen; and the immunization schedules for EAU in the various models were considerably different. It is likely therefore that differences in experimental design account for the variety of models already described, which indicates the importance of using well defined systems in producing experimental models such as these. Since the nature of the cellular infiltrate reflects the mechanism of the disease process, Marak et al. suggested that the polymorphonuclear cell response in high-dose EAU was due to complement activation via immune complex deposition, and provided evidence for this by showing that the severity of the disease could be reduced and the composition of its inflammatory cell exudate altered by depleting the animals of complement using cobra venom factor (otherwise known as C3 inactivator). In contrast, low-dose EAU was characterised by mononuclear cell infiltration only, and under these conditions, a cell-mediated autoimmune mechanism was proposed. Support for this view was provided by the demonstration that most of the signs of EAU could be eliminated by the administration of Cyclosporin-A, which is predominantly an inhibitor of T helper cells, and also by the observation that low-dose EAU could be transf erred with sensitized lymphocytes to naive animals (Forrester and Borthwick, unpublished observations). In the present study, both high- and low-dose disease were characterized predominantly by a mononuclear cell infiltrate, which suggests that both forms were T-cell-mediated. We have, however, observed a marked PMN response when crude homologous retinal extracts were used in the guinea pig. The preferential damage to ROS seen in this study with both low and high doses of S-antigen suggests that the ROS was the target for autodestruction in this model. Since S-antigen is chiefly located in the ROS, such a mechanism is predictable. Selective photoreceptor loss has only rarely been described before in S-antigen-induced EAU, although similar changes have been reported in models of rhodopsin-induced EAU. Although the model of Brown et al. is not strictly comparable to the present study, viz subconjunctival injection of S-antigen in complete Freund's adjuvant in rabbits, there was close morphologic similarity to the work reported here, ie, coagulative necrosis of rod outer segments, phagocytosis of ROS by macrophages, and hydropic degeneration and necrosis of inner segments. However, these workers also noticed proliferation of the RPE, whereas in the present study the most common change in the RPE was progressive atrophy. If the ROS is the target organ in EAU, then it is likely that the macrophage is the effector cell since only rarely were other cell types observed in the ROS layer. The exact mode of attack remains to be elucidated since both direct macrophage attack or antibody-dependent cytotoxicity are possible. However, it is also possible that the ROS is not the target cell but that it is damaged as the result of a "bystander response," resulting from release of nonspecific mediators from cytotoxic cells as is suggested to occur in experimental allergic encephalomyelitis. Important in this respect is the preponderance of eosinophils, particularly since IgE-mediated mechanisms have been implicated in the pathogenesis of EAU.

Whatever the mechanism, this ultrastructural study has made possible the identification of the ROS as the primary target in EAU. There have been few previous studies of the ultrastructural changes in EAU and of these only that by De Kozak et al. using crude ROS shows similarity to the present study.

Key words: S-antigen, uveoretinitis, ultrastructure, guinea pig

References

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