Experimental *Acanthamoeba* keratitis: II. Immunohistochemical evaluation

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Abstract

In a Wistar rat experimental model of *Acanthamoeba* keratitis immunohistochemical techniques were used to analyse the host cellular response. The inflammatory cell profile was observed to change at intervals. In tissue sections the cellular response consisted of neutrophils on the first day but predominantly macrophages on the following days. Some T lymphocytes but no B lymphocytes were observed.

The free-living amoeba *Acanthamoeba* causes infection at two distinct sites. It was first demonstrated to cause granulomatous encephalitis: this occurs in chronically ill or immunocompromised patients and is usually fatal.[1] Infection of the cornea later became recognised and is now more frequently diagnosed. *Acanthamoeba* may be cultured from corneal scrape or biopsy specimens, or may be demonstrated in tissue sections. The amoeba causes painful blinding keratitis and, in contrast to encephalitis, is usually seen in healthy people who wear contact lenses.[2,3] *Acanthamoeba* keratitis typically has a chronic progressive course, with stromal infiltration and frequent failures of medical and surgical treatment.[4]

The reasons why this organism of low virulence causes progressive ocular infection in normal hosts, and why keratitis often responds poorly to agents shown in vitro to be effective against the cultured isolate are unknown. Study of the host immune response to *Acanthamoeba* infection is very limited because only corneal transplantation specimens are available for study; such patients have in most cases been intensively treated with anti-inflammatory agents prior to surgery. Consequently, available reports[4-6] describe the late stages of the disease, probably modified by drug therapy.

Most pathological reports describe an acute inflammatory cell infiltrate in the stroma, with in some cases a sparse added lymphocyte component.[4,5] The report on two patients by Mathers and colleagues was the first to define by immunohistochemical methods the inflammatory response in *Acanthamoeba* keratitis.[6] They found that the inflammatory cell population comprised 80–100% neutrophils and macrophages near the site of active corneal ulceration.

The immune response of the host is probably important in development of this infection, and an analysis of the inflammatory cell types within the cornea subsequent to infection provides further understanding of the mechanisms of disease. A rat model of experimental *Acanthamoeba* keratitis has been established in which the infection is induced by intrastromal injection of the cyst form of *Acanthamoeba*.[7] Untreated tissue can be studied pathologically at specified intervals after disease induction. Conventional histological techniques and immunohistochemical staining with monoclonal antibodies against cell markers have been used to characterise the constituent cell types.

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Materials and methods

ACANTHAMOEBA INOCULATION AND CLINICAL EXAMINATION

The detailed procedure for induction of experimental keratitis has been previously reported.[7] Briefly, an axenic cyst suspension of a human keratitis isolate (*A polyphaga*, strain Shi) was prepared at a concentration of 1×10^6/ml in Page's amoeba saline. 1 µl of this suspension was inoculated by intrastromal injection into the right cornea of outbred 125 g male Wistar rats under general anaesthesia. The concentration of this suspension was predetermined as the dose necessary to induce reproducible clinical keratitis.

On days 1, 3, 5, 7, and weekly thereafter until 12 weeks following inoculation the rats were examined under general anaesthesia by slit-lamp. Corneal inflammation was graded on a scale of 0–3 for opacity (grade 0 being normal and grade 3 indicating dense corneal opacity), and the mean score for all infected corneas at each time interval was calculated. All procedures described conformed to the ARVO resolution on the Use of Animals in Research.

PREPARATION OF WAX SECTIONS

Two rats were killed by intraperitoneal injection of 60 mg pentobarbitone on days 1, 3, 7, 14, 21, 42, and 84 days after inoculation. The right eye was enucleated; the cornea was dissected and halved. One half was fixed for 24 h in neutral buffered formaldehyde, embedded in wax, and 6 µm sections cut with a microtome. Sections were stained, as previously described, with haematoxylin and eosin or rabbit anti-*Acanthamoeba* antibody (staining trophozoite or cyst forms brown).[7]

PREPARATION OF FROZEN SECTIONS

Hemicorneas from eyes enucleated as described above were mounted in embedding compound (Tissue-Tek OCT, Miles Scientific, Naperville, II) and snap-frozen in liquid nitrogen. Sections of 6 µm thickness were cut with a cryostat (2800 Frigocut-E, Reichert-Jung) at −30°C. Sections were mounted on polylysine-coated

Table 1 Monoclonal antibodies and incubations used in staining of corneas

Antibody	*Specificity*	*Dilution*	*Duration*	*Temperature*
ED2	Macrophages[8]	1:20,000	16 h	4°C
MRC OX-19	Pan-T cell[9]	1:100	16 h	4°C
MRC OX-33	Pan-B cell[10]	1:900	16 h	4°C
MRC OX-39	*Interleukin-2 receptor[11]	1:250	30 min	37°C
MRC OX-21	†Human C3b inactivator[12]	1:2	—	—

All monoclonal antibodies (Serotec Ltd, Oxford, England) were prepared from mouse hybridoma ascites fluid, except OX-21, which was prepared from tissue culture supernatant.
* Designating activated T-cells.
† Negative control, reacts with human but not rat tissue.

glass slides, air dried, immersed in acetone for 5 seconds, and frozen at −85°C for storage.

IMMUNOHISTOCHEMICAL STAINING OF FROZEN SECTIONS

Sections were stained by the peroxidase anti-peroxidase technique. Frozen sections were fixed in acetone for 10 min, air dried, and washed in distilled water for 30 min. Endogenous peroxidase activity was blocked by inoculation with 0·3% hydrogen peroxide for 30 min. After washing in phosphate-buffered saline (PBS), non-specific background staining was blocked by incubation with 1:5 normal rabbit serum in PBS for 30 min. Excess serum was blotted from sections, and mouse hybridoma monoclonal antibody was then added at specific dilutions, incubation times, and temperatures (Table 1). The sections were then washed and incubated with 1:50 secondary antibody (diluted with 1:10 normal rat serum) for 30 min at room temperature. Sections were again washed and incubated with 1:50 horseradish peroxidase anti-peroxidase (PAP) complex (diluted with 1:10 normal rat serum). After again washing with PBS, sections were incubated with a chromagen to identify antibody binding. For this purpose 3, 3′-diaminobenzidine (Sigma Chemical Co) was used, mixed with H_2O_2 in 0·1 M trometamol (Tris) buffer, giving a brown end product. After incubation for 7 minutes sections were washed in tap water for 5 minutes, counterstained with haematoxylin, and mounted.

Lymph node sections from normal Wistar rats, processed identically to the experimental sections, were used as positive controls. Lymph nodes contain a variety of inflammatory cell subtypes: thus node sections were moderately positive specimens, sensitive to variations in performance of the method. Experimental corneal sections for use as negative controls received, in place of primary antibody, an antibody which was known not to react with antigen in rat tissue. A monoclonal antibody against a human inflammatory product, MRC OX-21 (Serotec Ltd, Oxford, England), was used at dilution 1:2. This is a mouse monoclonal antibody against human C3b inactivator[12] and does not react with rat tissues.

QUANTITATIVE ANALYSIS OF SECTIONS

The positively stained inflammatory cells or *Acanthamoeba* in the stroma of the central cornea were counted with a graticule at 400× magnification. Three adjoining fields in each of two corneas from rats killed at the same interval were evaluated and a mean count calculated.

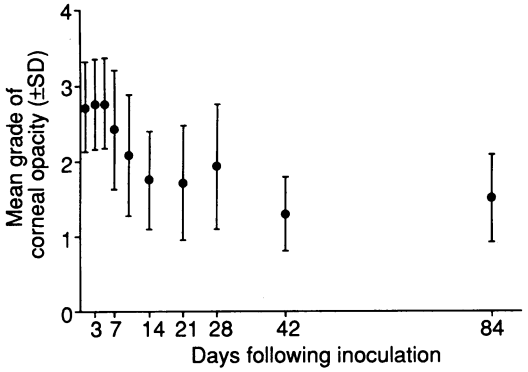

Figure 1 Grade of corneal opacity at intervals after induction of infection. The mean grade of opacity of all infected corneas examined on that day is shown.

Results

CLINICAL EVOLUTION OF KERATITIS

The inflammatory infiltration of the right cornea following *Acanthamoeba* inoculation as determined by slit-lamp examination is shown in Fig 1. Animals developed a stromal opacity which was granular in character. Corneal epithelial defects, vascularisation, perforation, and evidence of intraocular inflammation were not observed. Stromal opacity was maximal for the first seven days following inoculation and gradually declined after that time.

IMMUNOPEROXIDASE STAINING OF ACANTHAMOEBA IN TISSUE SECTIONS

Numbers of staining amoebae are shown in Table 2. An increasing proportion of trophozoite *Acanthamoeba* was observed in sections at intervals following inoculation.

CELLULAR COMPOSITION OF THE INFLAMMATORY INFILTRATE

Inflammatory cell types within the central stroma were identified by haematoxylin and eosin staining of neutrophils and by monoclonal antibody staining of surface antigens on macrophages, B cells, T cells, and activated T cells (Table 1). The relative number and proportions of inflammatory cells are shown in Table 3 and Figs 2A, B.

On day 1 neutrophils were the only inflammatory cell identified. A negligible number of other cell types staining with haematoxylin and eosin, which did not appear to be neutrophils, were not identified by staining with any of the monoclonal antibodies. On day three a similar total number of cells were seen but a proportion of these were macrophages.

Table 2 Acanthamoeba *in central corneal stroma*

Day	*Number of amoebae (mean)*	*SE*
1	8·7	1·5
3	12·9	1·3
7	6·1	0·6
21	2·0	0·7
42	0·6	0·3
84	1·3	0·3

Trophozoite and cyst forms of *Acanthamoeba* were stained by immunoperoxidase technique using rabbit anti-*Acanthamoeba* antibody. SE=standard error.

Table 3 Inflammatory cell types in the central stroma of corneas infected by Acanthamoeba

Day	Neutrophils cells	%	ED2+ cells	%	OX-33+ cells	%	OX-19+ cells	%	OX-39+ cells	%	Total
1	12 (1·0)	100	0 —	—	0 —	—	0 —	—	0 —	—	12
3	10 (1·4)	77	3 (0·8)	23	0 —	—	0 —	—	0 —	—	13
7	6 (0·5)	15	24 (6·3)	63	0 —	—	8 (2·7)	22	0 —	—	38
14	6 (1·0)	11	38 (1·3)	68	1 —	4	11 (2·3)	20	5 (1·4)	9	56
21	1 (0·6)	3	20 (2·7)	69	0 —	—	8 (1·0)	28	3 (0·8)	10	29
42	1 (0·3)	17	3 (0·5)	50	0 —	—	2 (0·4)	33	1 (0·5)	17	6
84	1 (0·1)	14	4 (1·0)	57	0 —	—	2 (1·6)	29	0 —	—	7

Cell counts and percentage proportions of inflammatory cells in the central stroma of corneas infected by *Acanthamoeba*. Neutrophils were stained with haematoxylin and eosin. Other inflammatory cell types were stained with monoclonal antibodies ED2 (macrophages), OX-33 (B cells), OX-19 (T cells), and OX-39 (activated T cells). Positively stained cells were counted with a graticule at 400× magnification. Standard error of the cell count in three fields in each of two corneas is given in parentheses. The total cell number given excludes OX-39+ (activated T) cells, which are taken to be a subset of OX-19+ (pan T) cells.

By day 7 macrophages had become the predominant cell type, constituting 63% of all cells. By this time neutrophils had sharply declined in absolute numbers and proportion (15%). T cells first appeared on day 7.

On day 14 the total cell count was highest. Macrophages continued to be the predominant cell type (Fig 3A). T cells constituted 20% of cells, and activated T cells were identified for the first time (Fig 3B). This indicates that the OX-19+ (pan-T) cells observed in earlier sections are probably not specifically directed against *Acanthamoeba* antigen.

On days 21 and 42 neutrophils continued to decline in numbers; macrophages continued to be the predominant constituent inflammatory cell; T cells and activated T cells continued to be observed in the same proportions. By day 42 the total number of inflammatory cells had markedly declined. On day 84 macrophages and T cells (OX-19+) persisted as the predominant cells in a sparse inflammatory infiltrate. Activated T lymphocytes (OX-39+) were no longer identified.

Almost no B lymphocytes were observed at any time, with only a solitary cell being stained, on day 14 (Fig 3C).

Discussion

The extensive use of hybridoma technology has led to the development of new methods for the isolation of murine immunoglobulins from cell culture or ascites fluids.[13] Monoclonal antibodies reactive with cell surface antigens have become the primary tools for study of inflammatory cell, and particularly lymphocyte, heterogeneity. Surface markers have rarely been found to be absolutely specific for any one inflammatory cell type. However, the specificity for rat inflammatory cells reported for those antibodies used in this study has led to their wide use in immunocytochemistry. Antibodies specific for rat T cell individual subsets are not commercially available at the time of writing.

Immunohistochemical study of the immune response in *Acanthamoeba* keratitis is necessary because so many features of this infection suggest an unusual host response to the pathogen. These include poor response to amoebicidal drugs,[4] chronic clinical course, lack of corneal vascularisation in human and experimental disease,[6 7] and lack of lymphocyte response in human pathological studies.[5 6]

In experimental *Acanthamoeba* keratitis we have identified a dynamic process in which the inflammatory cell profile alters with time. The inflammatory cell population was observed to be entirely neutrophil on day 1, with macrophages becoming the predominant cell type by day 7. T cells, including activated T cells, were constituents of the immune response after the first week; B cells were conspicuously absent. The numerical increase in amoebae seen at day 3 may be due to excystment and trophozoite multiplication. Decline after this time probably reflects destruction by inflammatory cells. While the clinical severity of keratitis was maximal in the first week, and the number of *Acanthamoeba* in tissue sections appeared maximal at day 3, the number of inflammatory cells was highest at day 14. These findings may indicate that amoebic destruction by the rat inflammatory response was maximal at around day 14. Destruction of amoebae was observed in sections at various time points but not quantified.

Immunohistochemical data from our study form an interesting contrast with those from two human corneal transplantation specimens reported by Mathers and colleagues.[6] These authors found that 80% to 100% of cells near the site of corneal ulceration were neutrophils or macrophages; no B or T cells were found in one cornea, and T cells constituting 10% with no B cells in the other. In both human and animal disease the reason for the absence of B cells in the inflammatory cell infiltrate is uncertain. It is unlikely that lack of corneal vascularisation explains the absence of B cells. In a model of experimental keratitis in the rat induced by

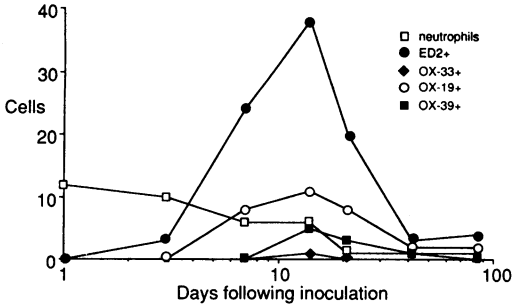

Figure 2A Differential inflammatory cell counts in infected corneas at intervals after inoculation of Acanthamoeba. *Horizontal axis is log scale.*

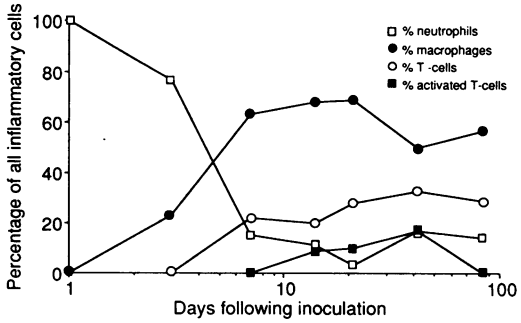

Figure 2B Differential inflammatory cell percentage proportions. Horizontal axis is log scale.

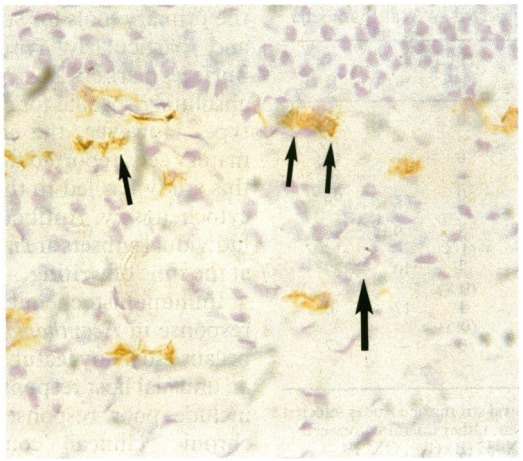

Figure 3A

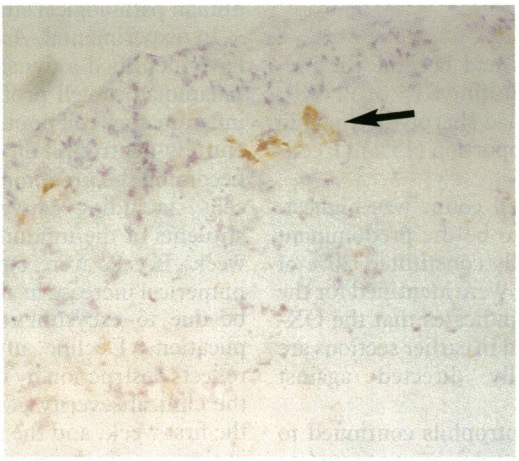

Figure 3B

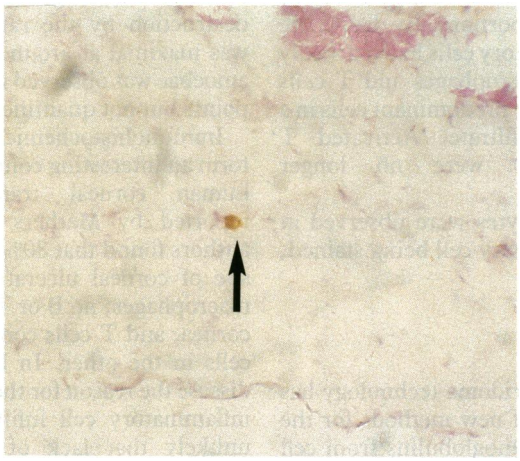

Figure 3C

Figure 3 Frozen sections of rat central cornea 14 days after inoculation with Acanthamoeba. *The sections were stained with monoclonal antibody: the brown colour indicates positive staining and the light purple colour is haematoxylin counterstain. (A) ED2 staining of a number of cells (small arrows) around* Acanthamoeba *cysts (large arrow) (×270). (B) MRC OX-39 staining of activated T cells (arrow) in anterior stroma (×170). (C) A solitary MRC OX-33 staining B cell (arrow) in deep stroma (×170). This was the only cell observed in any section which stained with this antibody.*

intrastromal injection of heat inactivated rabbit serum Verhagen and colleagues observed corneal vascularisation but no B lymphocytes in the inflammatory infiltrate.[14] In experimental *Acanthamoeba* infection it is, however, possible that there is systemic production of antibody without plasma cell or B cell infiltration into cornea. In this study we did not examine the serum for a measurable antibody response, which has been found in human infection.[15]

Immunological tolerance, the acquisition of non-reactivity towards particular antigens, may independently involve B and T cells and might explain the absence of B lymphocytes in the cornea. It might be that B cells are immature in the 6–8-week old rat, and that B cells encountering antigen for the first time become tolerant. Tolerance might also be mediated by soluble factors from suppressor T cells, and antigen-specific suppressor T cell-induced tolerance has been observed in mice.[16] Finally, as normal B cell responses to T-dependent antigens require help of specific T cells, reduced production of B cell maturation lymphokine IL-4 by the H_1 category of helper T cells might cause functional B cell deletion.

Further studies are necessary to characterise more comprehensively the immune response to *Acanthamoeba*. We plan to survey infected rat cornea for T cell subsets and class II major histocompatibility complex (MHC) antigens which would indicate local presentation of antigen. It would also be informative to extend immunohistochemical study to the limbus and local lymph nodes.

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