Background/aims: Effector T cell activation is particularly important in the initiation of autoimmune uveitis. This pilot study seeks to demonstrate activation of human peripheral effector T cells in response to the uveitis candidate autoantigen, retinal S antigen (SAg), using cytokine flow cytometry (CFC).

Methods: Peripheral blood mononuclear cell (PBMC) suspensions from uveitis patients and controls were stimulated with bovine SAg. Activation responses were detected by CFC.

Results: Electronic gating enabled analysis of CD69+, IFN-γ+ CD4+ lymphocytes. An SAg specific response was detectable in four of 13 patients and four of eight controls.

Conclusion: SAg specific, peripheral, effector T cell activation can be detected by CFC. Similar levels of responsiveness were seen in patient and control groups. More detailed cytokine profiling may demonstrate functional differences between the groups.

Materials and methods
Thirteen uveitis patients and eight clinically normal laboratory workers (without previous exposure to SAg) were recruited consecutively from our outpatient clinic (Table 1). Informed consent was obtained from each subject, and the study was conducted in compliance with the Declaration of Helsinki and with local ethics committee approval.

Ex vivo peripheral blood mononuclear cell (PBMC) stimulation and cytokine staining were broadly as described by Waldrop, but with a longer incubation time (see below). Experimental PBMC samples were incubated with 50 µg of bovine SAg and 1 µg anti-CD28 monoclonal antibody, while anti-CD28 alone was used in corresponding negative controls. Positive control samples were stimulated with 20 ng/ml phorbol myristate acetate (PMA), and 1 µM ionomycin. Brefeldin A was added to all samples at a final concentration of 10 µg/ml after 1 hour. Cells were stained for expression of CD4, CD8, CD69, and IFN-γ. Isotype matched monoclonal antibodies were used to evaluate background staining in positive control samples.

Abbreviations: CFC, cytokine flow cytometry; EAU, experimental autoimmune uveoretinitis; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; PMA, phorbol myristate acetate; SAg, retinal S antigen
An extended (18 hour) incubation was used, as higher antigen specific-negative control responder ratios were found with this modification (see Fig 1). No significant loss of CD4+ cells was noted as a result of extended incubation or brefeldin A toxicity, as previously noted. Data were analysed using WinMDI 2.8 software (http://facs.scripps.edu/software.html). Electronic gating on the negative control samples enabled identification of CD4+ (CD8−) lymphocytes. CD4+ T cells co-expressing IFN-γ and CD69 were identified in antigen specific and negative control samples. Quadrant markers were set around the negative control for IFN-γ expression and at 10^2 log fluorescence units for CD69. Results were analysed both in terms of individual responses and the aggregate responses of the patient versus control groups. In both cases the number of CD69+, IFN-γ+ events (per 100 000 CD4+ T cells) from each negative control sample, was subtracted from the corresponding number of antigen specific events. Individual samples

<table>
<thead>
<tr>
<th>Patient No</th>
<th>Clinical diagnosis</th>
<th>Activity</th>
<th>Duration of current episode</th>
<th>Total disease duration</th>
<th>Systemic therapy</th>
<th>Response to SAg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Intermediate uveitis</td>
<td>Inactive</td>
<td>NA</td>
<td>1 year</td>
<td>None</td>
<td>Negative</td>
</tr>
<tr>
<td>2</td>
<td>Panuveitis</td>
<td>Moderate</td>
<td>&gt;3 months</td>
<td>6 years</td>
<td>Tacrolimus 7 mg/day</td>
<td>Positive</td>
</tr>
<tr>
<td>3</td>
<td>Posterior uveitis</td>
<td>Mild</td>
<td>&gt;3 months</td>
<td>11 months</td>
<td>None</td>
<td>Negative</td>
</tr>
<tr>
<td>4</td>
<td>Posterior uveitis</td>
<td>Severe</td>
<td>&lt;3 months</td>
<td>1 month</td>
<td>None</td>
<td>Positive</td>
</tr>
<tr>
<td>5</td>
<td>Intermediate uveitis</td>
<td>Mild</td>
<td>&gt;3 months</td>
<td>11 years</td>
<td>None</td>
<td>Negative</td>
</tr>
<tr>
<td>6</td>
<td>Intermediate uveitis</td>
<td>Mild</td>
<td>&gt;3 months</td>
<td>13 months</td>
<td>None</td>
<td>Positive</td>
</tr>
<tr>
<td>7</td>
<td>Posterior uveitis</td>
<td>Moderate</td>
<td>&gt;3 months</td>
<td>17 years</td>
<td>None</td>
<td>Negative</td>
</tr>
<tr>
<td>8</td>
<td>Intermediate uveitis</td>
<td>Mild</td>
<td>&gt;3 months</td>
<td>8 months</td>
<td>None</td>
<td>Negative</td>
</tr>
<tr>
<td>9</td>
<td>Intermediate uveitis</td>
<td>Mild</td>
<td>&gt;3 months</td>
<td>2 years, 10 months</td>
<td>None</td>
<td>Negative</td>
</tr>
<tr>
<td>10</td>
<td>Panuveitis</td>
<td>Moderate</td>
<td>&gt;3 months</td>
<td>18 years</td>
<td>None</td>
<td>Positive</td>
</tr>
<tr>
<td>11</td>
<td>Posterior uveitis</td>
<td>Mild</td>
<td>&gt;3 months</td>
<td>5 years</td>
<td>Cyclosporin 150 mg/day</td>
<td>Equivocal</td>
</tr>
<tr>
<td>12</td>
<td>Intermediate uveitis</td>
<td>Mild</td>
<td>&gt;3 months</td>
<td>2 years</td>
<td>None</td>
<td>Negative</td>
</tr>
<tr>
<td>13</td>
<td>Intermediate uveitis</td>
<td>Inactive</td>
<td>NA</td>
<td>3 years, 3 months</td>
<td>None</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Figure 1  Increased CD4+ responder levels are seen with extended incubation, without significant loss in overall CD4+ T cell frequencies. (A, B) Negative control samples of 6 and 18 hours incubation respectively. (C, D) Antigen stimulated samples, 6 and 18 hours respectively. CD4+ cells as a percentage of total events are as follows: (A) 35.5% (B) 31.8% (C) 36.1% (D) 33.6%. Increased background subtracted response rates (CD69+, IFN-γ+) are seen with extended [18 hour] antigen stimulation, without a significant decrease in the proportion of overall CD4+ T cells.
were categorised as follows: >10 events per 100 000, “positive”, 5–10 events, “equivocal”, <5 events, “negative.” Aggregate patient and control results were compared using an unpaired two tailed t test with Welch’s correction, and the result expressed as a p value (GraphPad Prism).

RESULTS

Our main finding is that antigen specific effector T cell activation was detected in both uveitis patient and control groups. Analysis of individual samples showed four out of 13 patient samples responding positively to the SAg/anti-CD28 stimulus, with one further patient’s response being equivocal. Four out of eight controls also responded positively. A typical positive response evident. Total number of PBMCs analysed: A and B, 50 000; C and D, 500 000.

DISCUSSION

This pilot study was designed to establish the feasibility of detecting autoantigen specific effector T cell activation in autoimmune uveitis or control samples using the technique of CFC. We are confident that we have demonstrated this in samples from both groups of subjects. Production of IFN-γ by responding cells may indicate a Th1 type response to SAg. Our study confirms the suitability of CFC for testing the immunogenicity of candidate autoantigens in uveitis, and analysing rare event cytokine responses in non-naïve effector T cells.

Flow cytometry has previously been used to determine the expression of cell phenotypic markers/intracellular cytokines in unstimulated ocular cells and PBMCs in autoimmune uveitis.\textsuperscript{12–13} CFC combined with non-specific stimulation has been used to detect increased frequencies of IFN-γ and IL-4 producing PBMCs in Behçet’s disease.\textsuperscript{17} and increased levels of CD69 expression in parallel with disease activity.\textsuperscript{18} The use of CFC combined with stimulation by known uveitis autoantigens has even greater potential for advancing our understanding of in vivo disease mechanisms.

In addition to uveitis patients, a similar proportion of healthy controls responded to stimulation by SAg. Responses to SAg have been noted previously in normals using other techniques,\textsuperscript{12–13} and our results would appear to confirm this phenomenon. Notably there was no significant difference detected between the groups in the aggregate levels of responsiveness, albeit using a small sample. Differences might become more apparent with larger sample sizes.

Qualitative differences in cytokine profiles expressed by activated cells in uveitis patient and control groups may well be as relevant to disease progression as overall frequency of cell activation. Modern multiparameter flow cytometers,
capable of accurate Th1 versus Th2 profiling of activated cells, would be able to detect any such differences. Cytokine profiling, in responses to stimulation by nominal uveitis autoantigens, at different levels of disease activity, or in the evaluation of immunotherapies, would provide invaluable insights into uveitis mechanisms.

This study demonstrates that responses to retinal autoantigens are detectable using CFC, and that these are both specific and sensitive. We hope to see the full potential of this technique being explored in human autoimmune uveitis in the future.

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