

SCIENTIFIC CORRESPONDENCE

S antigen specific effector T cell activation detected by cytokine flow cytometry

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Br J Ophthalmol 2002;**86**:517–520

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.

Autoimmune uveitis¹ is a CD4+ T cell mediated, ocular inflammatory condition that causes damage to the choroid and retina with resultant visual loss. Several retinal proteins have been proposed as potential autoantigens in autoimmune uveitis,^{2–7} mainly on the basis of their ability to induce experimental autoimmune uveoretinitis (EAU) in certain strains of laboratory animal. Probably the most widely studied is retinal S antigen (SAg), a 48 kD protein found in the rod outer segment.⁸

SAg has previously been shown to stimulate T cell proliferation in animal and human cells, both in autoimmune uveitis patients^{9–11} and healthy controls.^{12–13} However, proliferation based assays are mostly relevant to clonal expansion of memory T cells, and tell us little about other outcomes, particularly activation of preprimed, effector T cells. The effects of T cell “activation,” meaning the expression of cytokines and/or cell surface markers on effector cells, can dictate the whole direction of the autoimmune response. Where the predominant cytokines produced by activated CD4+ cells are IFN- γ , TNF- α , or IL-2 (a Th1 type response), it is likely there will be a delayed type hypersensitivity reaction.¹⁴ Where IL-4, IL-5, or IL-6 predominate (Th2 response), tissue destruction is less likely and may even be suppressed.

To study SAg specific, effector T cell activation in a rare cellular subgroup such as the autoreactive CD4+ population in autoimmune uveitis, a *non*-proliferation based technique for studying cytokine and surface marker expression at the single cell level is needed. Of existing single cell techniques ELISPOT, limiting dilution, T cell cloning, single cell polymerase chain reaction (PCR), and in situ hybridisation are either proliferation based, require laborious preselection or extended incuba-

tion of cells. The technique of cytokine flow cytometry (CFC)¹⁵ could potentially circumvent many of these limitations.

CFC has emerged as an ideal technique for analysing cytokine production in activated lymphocytes. Because it is a single cell technique, it enables detection and enumeration of cytokine responses from rare T cell subgroups in unselected cell populations.¹⁶ Technically a relatively straightforward technique, CFC allows rapid analysis of large numbers of activated or non-activated cells. Multiparameter analysis, allowing simultaneous detection of several cytokines/surface markers, combined with electronic “gating” procedures, permits true Th1 versus Th2 differentiation for a specific T cell subpopulation. Furthermore, CFC allows the study of individual T cells directly *ex vivo*, minimising artefacts caused by extended (>24 hours) culture.

In autoimmune uveitis, CFC has to date mainly been used to study T cell responses to non-specific stimuli such as mitogens and ionophores.^{17–18} However, little has been reported on the use of CFC to detect T cell activation in response to stimulation by nominal uveitis autoantigens. CFC has great potential, not only in the direct testing of T cell reactivity to retinal autoantigens, but also in analysing Th1 versus Th2 cytokine differentiation. In this pilot study, we therefore sought to adapt this technique to the study of autoimmune uveitis, to demonstrate antigen specific effector T cell activation in response to stimulation by whole SAg.

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,¹⁹ but with a longer incubation time (see below). Experimental PBMC samples were incubated with 50 μ g of bovine SAg and 1 μ g 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 μ M ionomycin. Brefeldin A was added to all samples at a final concentration of 10 μ g/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

Table 1 Autoimmune uveitis patient details and responses to SAg

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

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.^{20, 21} 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

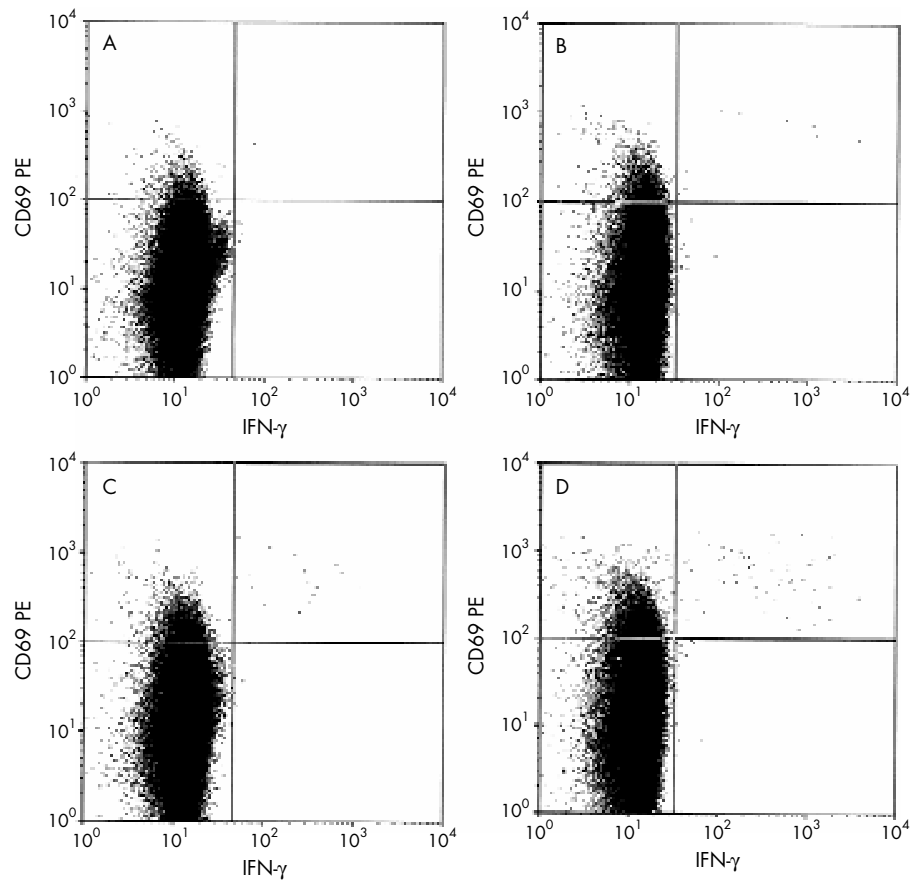


Figure 1 Increased CD4+ responder levels are seen with extended incubation, without significant loss in overall CD4+ T cell frequencies. (A, B) Negative control samples of 6 and 18 hours incubation respectively. (C, D) Antigen stimulated samples, 6 and 18 hours respectively. CD4+ cells as a percentage 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.

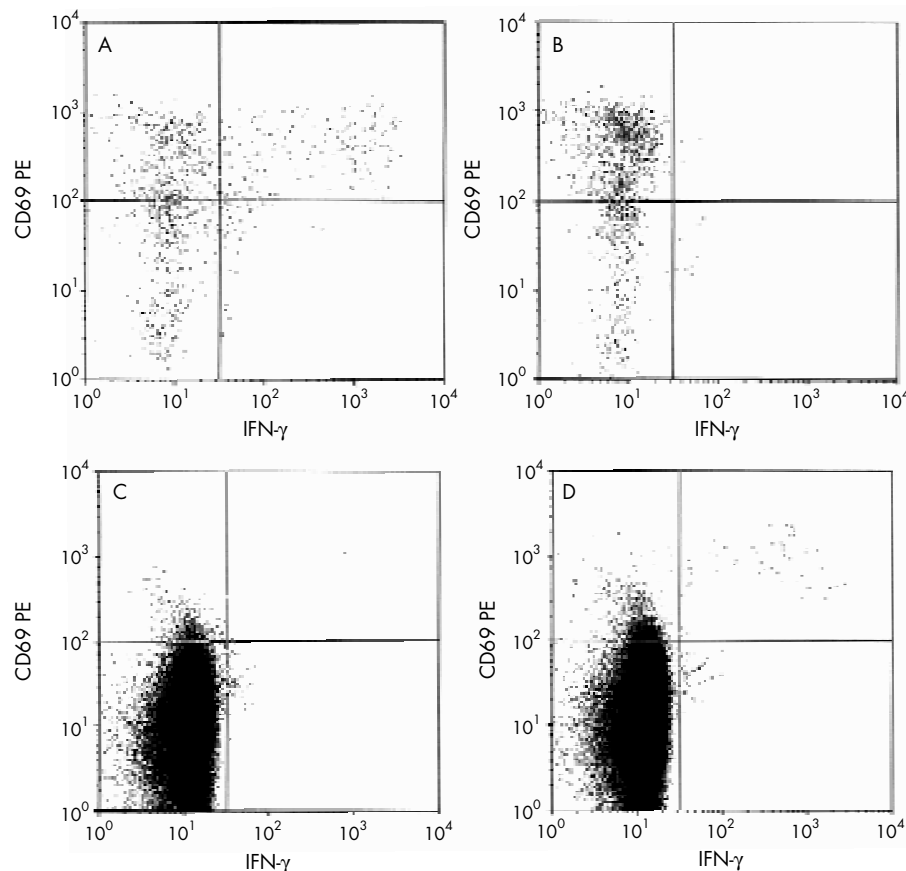


Figure 2 Typical antigen specific response to SAg in a uveitis patient sample (gated on CD4+ lymphocytes). (A) PMA/Ionomycin stimulated sample. (B) Same as (A), but with isotype matched negative control staining. Negligible levels of background staining noted. (C) Negative control: cells incubated in medium and anti-CD28 for 18 hours. (D) Antigen stimulation: cells incubated with SAg and anti-CD28 for 18 hours. Positive response evident. Total number of PBMCs analysed: A and B, 50 000; C and D, 500 000.

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 from a uveitis patient is shown in Figure 2.

Analysis of aggregate, baseline subtracted positive events in the uveitis group showed a mean of 7.98 events per 100 000 (95% CI, 0.62 to 15.34) and a mean of 9.37 (95% CI, 1.10 to 17.63) in the control group. However, *no* significant difference in responder frequency was detected between the groups (*p*=0.779). In the uveitis patient samples that demonstrated a response, the percentage of positive events ranged from 0.015% to 0.047% of the CD4+ T cell population. Analysis of positive control responses confirmed that cell samples had been immunologically competent. Negligible levels of non-specific background staining were noted. As previously reported,²² PMA and ionomycin stimulated samples showed substantial downregulation of surface CD4 expression.

Of the four positive “responders” in the uveitis group, one had intermediate uveitis, two (of two) had panuveitis, and one had posterior uveitis.²³ All had some active inflammation at time of sampling. One of the four was taking systemic immunosuppression.

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.^{24–26} 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,¹⁷ and increased levels of CD69 expression in parallel with disease activity.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,^{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.

ACKNOWLEDGEMENTS

This work was supported in part by the Wellcome Trust, London, UK and by Fight For Sight, London, UK. The authors thank Alison Galvin for technical assistance with flow cytometry analysis and April Powell-Richards for preparation of antigen.

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Accepted for publication 21 November 2001

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Br J Ophthalmol 2002 86: 517-520

doi: 10.1136/bjo.86.5.517

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