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Identification of T-cell stimulatory antigens of *Chlamydia trachomatis* using synovial fluid-derived T-cell clones

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SUMMARY

Chlamydia trachomatis is a major cause of sexually transmitted disease, infertility and reactive arthritis in the Western world, and of trachoma in the developing world. There is evidence that the chronic inflammatory reaction seen in diseases associated with chlamydiae represents a delayed-type hypersensitivity response to chlamydial antigens. Little is known about which chlamydial antigens elicit T-cell responses yet such information could have important implications in terms of both immunopathological understanding of these diseases and immunoprophylaxis design. In this study, 61 chlamydia-specific T-cell clones have been produced from the synovial fluid of an individual with sexually acquired reactive arthritis (SARA). Ten clones have been characterized in detail and used to identify T-cell stimulatory antigens of chlamydiae by means of T-cell immunoblotting. Two distinct antigenic fractions have been identified, one recognized by three of the clones (molecular weight 18,000), the other recognized by six of the clones (molecular weight 30,000). The fractions are distinct from the major outer membrane protein, the 57,000 MW stress protein and the 60,000 MW cysteine-rich membrane protein of chlamydiae. The major histocompatibility complex (MHC) restriction of the response to these antigens differed: clones recognizing the 18,000 MW antigen required antigen-presenting cells expressing DR1 subtype DRB1*0101 or DRB1*0102 which only differ at amino acids 85 and 86 on the DR β-chain; by contrast clones recognizing the 30,000 MW antigen were presented to only by antigen-presenting cells from DRB1*0101 individuals, reflecting extreme sensitivity of these clones to the polymorphism at positions 85 and 86 on the DR β-chain.

INTRODUCTION

Chlamydia trachomatis, an obligate intracellular bacterium, is an important pathogen of man, being a major cause of sexually transmitted disease in the Western world and, in developing countries, responsible for trachoma—the major infectious cause of blinding eye disease.[1,2] More recently it has been identified as an important cause of pelvic inflammatory disease leading to infertility,[3] and also of sexually acquired reactive arthritis (SARA).[4] Each of these diseases is a chronic inflammatory condition in which it is often not possible to culture chlamydial organisms from the site of disease. There is evidence to suggest, particularly in the case of trachoma, that the inflammatory response seen represents a delayed-type hypersensitivity reaction to chlamydial antigens. In experimental models of trachoma, it has been found that monkeys which have sustained a previous ocular chlamydial infection develop a trachoma-like condition if a solubilized chlamydial preparation is repeatedly instilled into their eyes.[5] Moreover, the same condition develops in guinea-pigs sensitized by previous infection, if the 57,000 MW chlamydial stress protein is instilled topically.[6]

SARA is a sterile inflammatory oligoarthritis occurring typically 7–20 days following an episode of genitourinary inflammation.[7] Evidence for the involvement of chlamydiae in SARA comes from both direct urethral culture of organisms from affected individuals and serological findings.[4,8] In some instances, chlamydial antigens have been identified by immunofluorescence staining of material from the inflamed joints of affected individuals,[9,10] and chlamydial DNA has been identified by use of polymerase chain reaction (PCR).[11] By contrast, chlamydiae have not been cultured from the joint. As with trachoma, there is strong evidence that SARA is an immunologically mediated disease. Histological analysis reveals a chronic inflammatory process in the synovium although the joint is sterile. The presence of chlamydia-specific T cells at the site of inflammation can be demonstrated by *in vitro* culture of T cells taken from the affected joint with optimal concentrations of chlamydiae, when a marked proliferative response is observed.[12,13]

The concept that disease associated with chlamydiae is immunologically mediated raises the question as to which

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chlamydial antigens the immune response is directed against. Whereas there has been considerable progress in identifying chlamydial antigens which induce antibodies,[14-17] little is currently known about the antigens that elicit T-cell responses. We report the use of synovial fluid-derived, chlamydia-specific T-cell clones from an individual with SARA to identify stimulatory chlamydial antigens by means of T-cell immunoblotting.

MATERIALS AND METHODS

Patient

The T-cell clones were derived from a 26-year-old male with chronic SARA for 6 years. He had suffered a seronegative inflammatory oligoarthritis the onset of which followed an episode of urethritis. *Neisseria gonorrhoea* was cultured from the urethra. Despite appropriate anti-gonococcal therapy the urethritis persisted and was treated with tetracycline as local experience had shown a concurrent chlamydial infection in 50% of individuals with gonorrhoea. No attempt to culture chlamydiae was made at this time. He subsequently suffered balanitis, conjunctivitis, persistent arthritis and further episodes of non-specific urethritis. At the time of aspiration he had a sterile synovitis in both knees. Anti-chlamydial antibodies were demonstrated in both serum and SF by immunoblotting. His HLA antigens included B27, DR1, DR3.

Proliferation assays

Assays of polyclonal synovial fluid and peripheral blood mononuclear cells. Mononuclear cells (MC) were obtained from heparinized synovial fluid (SF) and peripheral blood (PB) by centrifugation on Ficoll–Paque. Synovial fluid was first incubated with 10 U/ml hyaluronidase at 37° for 30 min. Cells were washed twice and resuspended in RPMI-1640 with 10% human serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin [complete medium (CM)]. Mononuclear cells (5×10^4) were incubated with antigens in CM for 6 days in 0·2 ml round-bottomed wells. Proliferation was assessed by [^{3}H]thymidine (0·15 μCi/well) uptake over the last 18 hr.

Assays of T-cell clones. For all except T-cell immunoblot assays, 2×10^4 cloned T cells were incubated with antigen and 5×10^4 irradiated (30 Gy) DR-matched PBMC in CM in 0·2 ml round-bottomed wells for 3 days with measurement of [^{3}H]thymidine uptake over the last 18 hr. T-cell immunoblot assays were carried out in the same way but in flat-bottomed 0·2 ml wells with 4×10^4 clone cells and 1×10^5 PBMC.

Antigens

Chlamydia trachomatis. Chlamydia trachomatis E/DK20/ON (strain DK20) elementary bodies (EB) were grown in McCoy cells and purified by the Urografin batch procedure.[18] Strain DK20 is an oculogenital strain and so was appropriate for this study. In the assays of SFMC and PBMC, 2×10^8/ml purified EB of strain DK20 were used. This preparation was also used to produce the T-cell blasts from which the chlamydia-specific clones were derived.

In the clone proliferation assays, either purified EB of strain DK20 or a solubilized preparation were used (as indicated in the text). The latter was obtained by solubilizing EB in sample buffer as described by Laemmli,[19] followed by dialysis for 48 hr in 1-litre phosphate-buffered saline (PBS) with four changes. The solubilized material was used at a protein concentration of 0·4 μg/ml.

Other antigens. In the assays of responses of the freshly isolated SFMC, the following preparations were used at previously defined optimal concentrations:[12] *Salmonella agona*, killed by γ-irradiation, 2×10^8/ml; heat-killed *Yersinia enterocolitica* O:3, 2×10^6/ml; purified protein derivative (PPD) of tuberculin (Statens Seruminstitut, Copenhagen, Denmark), at 20 μg/ml.

T-cell cloning

T-cell blasts were produced by 6 days of culture of SFMC with strain DK20 EB and then cloned by limiting dilution (1 or 0·5 T cells/well) in 20 μl Terasaki wells using 1×10^6/ml irradiated autologous mononuclear cells, 100 U/ml recombinant interleukin-2 (IL-2) (Cetus, Emeryville, CA) and the same chlamydial preparation. The clones were expanded at 21-day intervals by restimulation with 5×10^5/ml irradiated allogeneic PBMC, IL-2 (50 U/ml), 1 μg/ml phytohaemagglutin (PHA) (HA16; Wellcome Laboratories, Beckenham, U.K.) in CM supplemented with 1% non-essential amino acids and 1% sodium pyruvate. Antigen specificity was examined at least 10 days after restimulation with PHA. By this method, 79 T-cell clones were obtained, of which 61 were chlamydia specific on a screening proliferation assay. Chlamydial specificity was defined as a stimulation index > 3 (where stimulation index = [^{3}H]thymidine uptake in the presence of chlamydiae divided by [^{3}H]thymidine uptake in the presence of medium alone).

Preparation of nitrocellulose-bound chlamydial antigens[20]

Purified strain DK20 EB were solubilized in sample buffer[19] containing 2 mM phenylmethyl sulphonyl fluoride and separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (*c.* 260 μg protein/minigel). The separated proteins were electroblotted onto a 0·45μm nitrocellulose sheet (Schleicher & Schuell) which was then sliced horizontally into 24 strips so that each strip contained chlamydial protein of a certain molecular weight. The strips were washed twice in 10% ethanol/distilled water, and then four times in PBS. Strips were stored at −20° and γ-irradiated prior to use in clonal proliferation assays. For the clone assays, 5 mm lengths of each strip were used per 0·2 ml well as described above. Assays of each fraction of nitrocellulose-bound antigen were performed in duplicate or triplicate. Nitrocellulose spotted with PBS was used as a control.

MHC class II-specific monoclonal antibodies (mAb)

Antibodies specific for HLA-DP, -DQ and -DR, obtained from Becton Dickinson (Mountain View, CA), were used at dilutions of 1 in 100 and 1 in 200, to assess their effect on T-cell clone responses to chlamydia.

HLA-DR sub-typing

A 232 base pair (bp) segment of the first domain of all known DRB sequences was amplified from genomic DNA by PCR, using *Taq* polymerase (Perkin Elmer Cetus, Beaconsfield, U.K.)[21] and the primers GLPDR and GAMPDR (synthesized by Dr J. Fox, Alta Bioscience, University of Birmingham).[22] The product was blotted onto nitrocellulose filters (Hybond-C, Amersham International, Amersham, U.K.) using a Hybri-Dot manifold (Gibco-BRL, Uxbridge, U.K.). Radiolabelled allele-specific oligonucleotide probes (Table 1) were hybridized to the

Table 1. Oligonucleotide probes specific for the DRB1 alleles, used for HLA-DR typing. The probes are named on the basis of their classic HLA DR specificity. The amino acid positions coded by the probe in the mature sequence are indicated. The phenotype is inferred from the combination of probes bound as shown on the right of the table

Probe	Sequence	AA POS'N	DR allele B1*0101	B1*0102	B1*0103	B1*0301/2
	5′ 3′					
DR1	G GAA AGA TGC ATC TAT A	27–33	+	+	+	−
DR3	GGC CGG GTG GAC AAC TA	73–78	−	−	−	+
Dw10	GAA GAC GAG CGG GCC GCG	69–74	−	−	+	−
Dw14	GAG CAG AGG CGG GCC GCG	69–74	+	+	−	−
Dw20	TAC GGG GCT GTG GAG AG	83–88	−	+	−	−

(+) PCR product from DR allele binds probe.

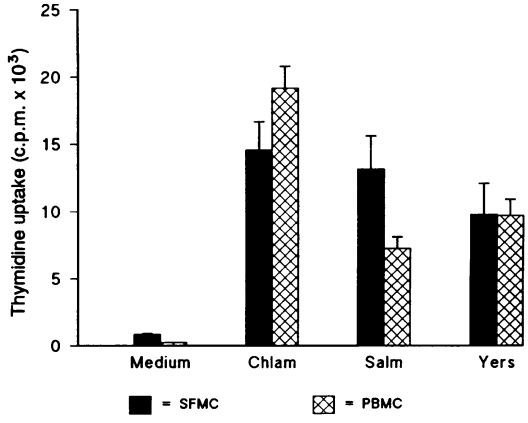

Figure 1. Proliferative responses ([^{3}H]thymidine incorporation, c.p.m.) (mean of three observations) of 5×10^4 SFMC or 5×10^4 PBMC incubated for 6 days with medium alone, chlamydial strain DK20 EB, or optimal concentrations of killed *S. agona* and *Y. enterocolitica* O:3. Bars represent SE.

PCR product using the method of Wordsworth *et al.*[23] DR phenotype was inferred from the combination of probes bound (Table 1).

Statistical analysis

Mean polyclonal proliferative responses to different antigenic preparations were compared using Student's *t*-test.

RESULTS

Synovial fluid and peripheral blood mononuclear cell responses to reactive arthritis organisms

PB and SF were obtained from a 26-year-old man with chronic SARA. The PBMC and SFMC proliferative responses to a panel of bacteria associated with reactive arthritis were measured (Fig. 1). Prominent proliferative responses to *C. trachomatis* were evident within both SFMC and PBMC. There were also significant responses to preparations of *Y. enterocolitica* and *S. agona*, organisms which can also be associated with reactive arthritis; however the PBMC response to *C. trachomatis* was significantly greater than the response to the other two organisms ($P < 0{\cdot}05$). In a patient with typical clinical features of reactive arthritis beginning shortly after a sexually acquired

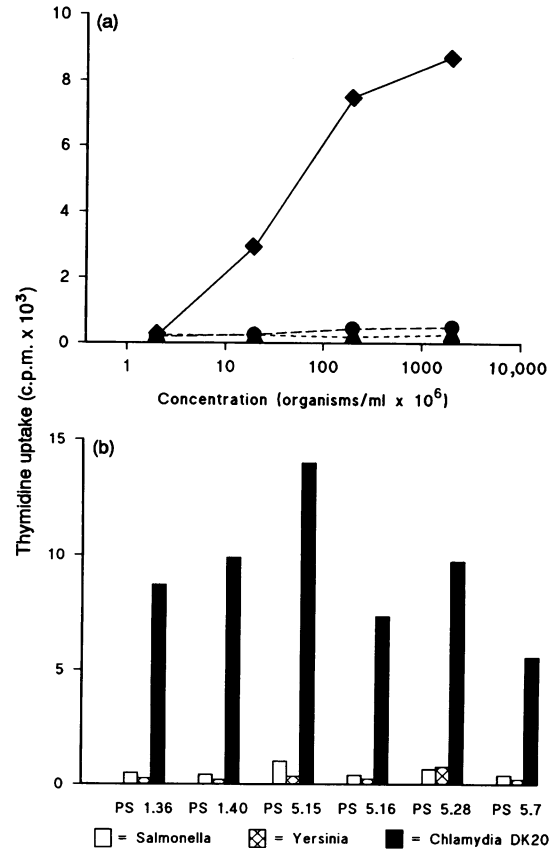

Figure 2. (a) Proliferative responses ([^{3}H]thymidine incorporation, c.p.m.) of 2×10^4 clone PS 1.36 T cells incubated for 3 days with 5×10^4 irradiated DR-matched PBMC as antigen-presenting cells and a range of dilutions of *Y. enterocolitica* (●), *S. agona* (▲) and *C. trachomatis* (◆). (b) Proliferative responses of six clones to the same organisms are shown. For each clone, responses were assessed to the same range of dilutions as in (a). Maximal responses to each organism are shown.

infection, and in whom chlamydia-specific antibodies were demonstrated by immunoblotting, these results were highly suggestive of reactive arthritis associated with *C. trachomatis*, and similar to those seen in patients in whom the diagnosis has been confirmed by culture of the organism.[12,13]

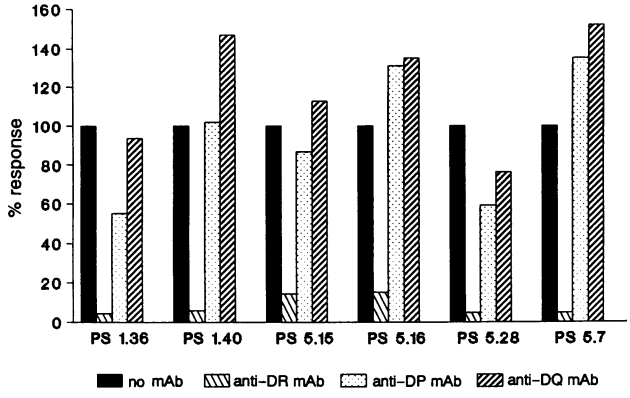

Figure 3. Proliferation of 2×10^4 T cells of each of six clones in response to 1·1 μg/ml solubilized chlamydial strain DK20 EB, presented by 5×10^4 irradiated autologous PBMC. Responses were measured in the presence of mAb specific for HLA-DR, -DP and -DQ, each at a dilution of 1 in 100. Responses are shown as a percentage of the response to chlamydial antigen in the absence of mAb. Responses were measured as described in the legend to Fig. 2.

T-cell clone specificities

T-cell blasts obtained by a 6-day incubation of SFMC with strain DK20-purified EB were cloned by limiting dilution. Seventy-nine T-cell clones were produced from wells seeded at 0·5 or 1 cell/well. Of these, 61 were chlamydia specific (stimulation index > 3) on an initial screening assay. Six of those clones showing the largest response to chlamydia were fully characterized and the antigen specificity of a further four also assessed. All were $CD4^+$, $CD8^-$, T-cell receptor-δ^-, by mAb staining and flow cytometry (data not shown). A typical example of clone responses to a range of dilutions of *C. trachomatis*, *Y. enterocolitica* and *S. agona* is shown in Fig. 2a. Figure 2b shows the results of the same dose–response experiment for a further five clones. In these cases the maximal response to each preparation is shown. All six clones showed significant responses to chlamydia but did not respond to yersinia or salmonella. No clone demonstrated any proliferative response to a range of dilutions of sonicated McCoy cells uninfected with chlamydia, confirming that the clones were recognizing an antigen which was chlamydial in origin (data not shown).

Clone major histocompatibility complex (MHC) restriction

The effect on chlamydia-induced proliferation of the addition of major histocompatibility complex (MHC) class II-specific mAb was examined (Fig. 3). In all cases the response was grossly diminished by the addition of HLA-DR-specific mAb, but was little affected by HLA-DP- or HLA-DQ-specific mAb.

To determine the exact MHC restriction of the clones, experiments were performed using irradiated PBMC from normal donors of known DR type to present chlamydial antigen to the clones (Table 2 shows the result for six of the clones). The patient from whom the clones were derived was $HLA\text{-}DR1^+$ (subtype DRB1*0101), $DR3^+$. None of the clones recognized antigen presented by $DR3^+$ cells, rather all clones recognized antigen presented by cells which were $DRB1*0101^+$. Experiments using presenting cells of different DR1 subtypes showed that clones PS 1.40, 5.15, and 5.7 also recognized antigen presented by cells of DR1 subtype DRB1*0102 whereas the other three clones did not. None of the clones responded to chlamydial antigen presented by cells of DR1 subtype DRB1*0103.

Identification of chlamydial antigens

Antigens from solubilized DK20 EB were separated and transferred to nitrocellulose as described in Materials and Methods (Fig. 4). The antigens recognized by the chlamydia-specific clones were identified by means of T-cell immunoblotting. Figure 5 shows a representative example of the response of each of the six clones to the same preparation of nitrocellulose blotted with PAGE-fractionated solubilized strain DK20. Clones PS 1.40, 5.15 and 5.7 consistently recognized a chlamydial fraction of approximately 18,000 MW, while clones PS 1.36, 5.16 and 5.28 recognized a fraction of approximately 30,000 MW. No clone recognized both chlamydial fractions. The antigen specificity of four further clones was assessed. All were

Table 2. The ability of each of the six clones to proliferate (in a 3-day assay) in response to chlamydial strain DK20 EB presented by PBMC of normal donors of known HLA-DR type. The HLA DR-B1 subtype of the individuals tested is shown in parentheses. Donor DP was DR3 homozygous

Presenter (DR-B1 subtype)	PS 1.36	PS5.16	PS5.28	PS1.40	PS5.15	PS5.7
KG(*0101)	+	+	+	+	+	+
JW(*0101)	+	+	+	+	+	+
MS(*0101)	+	+	+	+	ND	+
SD(*0101)	+	+	+	+	+	+
CG(*0102)	−	−	−	+	+	+
AD(*0102)	−	−	−	+	ND	+
KR(*0103)	−	−	−	−	ND	−
DP(DR3)	−	−	−	−	−	−

(+) Clone response to chlamydia (SI > 10).
(−) No clone response to chlamydia (SI < 2).
ND, Not done.

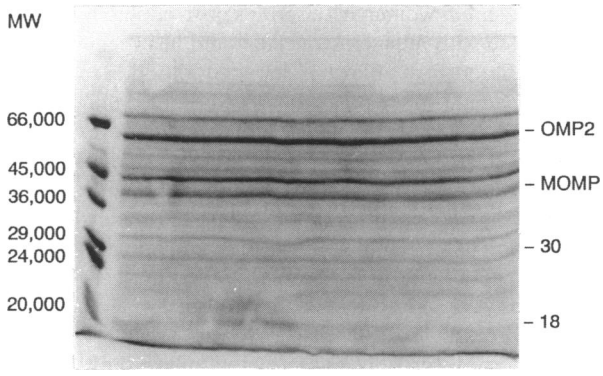

Figure 4. Photograph of PAGE separated solubilized chlamydial proteins blotted onto nitrocellulose and stained with Amido black. The position of MW markers is shown on the left; bands corresponding to chlamydia antigens OMP2, MOMP, and the 30,000 and 18,000 MW antigens identified by T-cell clones are shown on the right.

HLA-DR1 restricted. Three of these clones recognized the 30,000 MW fraction (data not shown); the fourth did not respond to any of the nitrocellulose fractions. Clone responses were highly reproducible, both in repeated experiments using the same nitrocellulose preparation, and in experiments using new preparations of nitrocellulose-blotted chlamydial antigens. In addition, testing the uncloned SFMC by T-cell immunoblotting revealed proliferative responses to both of the fractions identified by the T-cell clones, with responses to other fractions also evident (data not shown).

DISCUSSION

There is evidence that the chronic inflammatory reaction which characterizes diseases caused by *Chlamydia trachomatis* represents a delayed-type hypersensitivity response to chlamydial antigens. The identification of such antigens is therefore of great relevance. Our strategy, to isolate chlamydia-specific T-cell clones from a site of inflammation and use them in T-cell immunoblotting, has successfully identified two distinct chlamydial antigens.

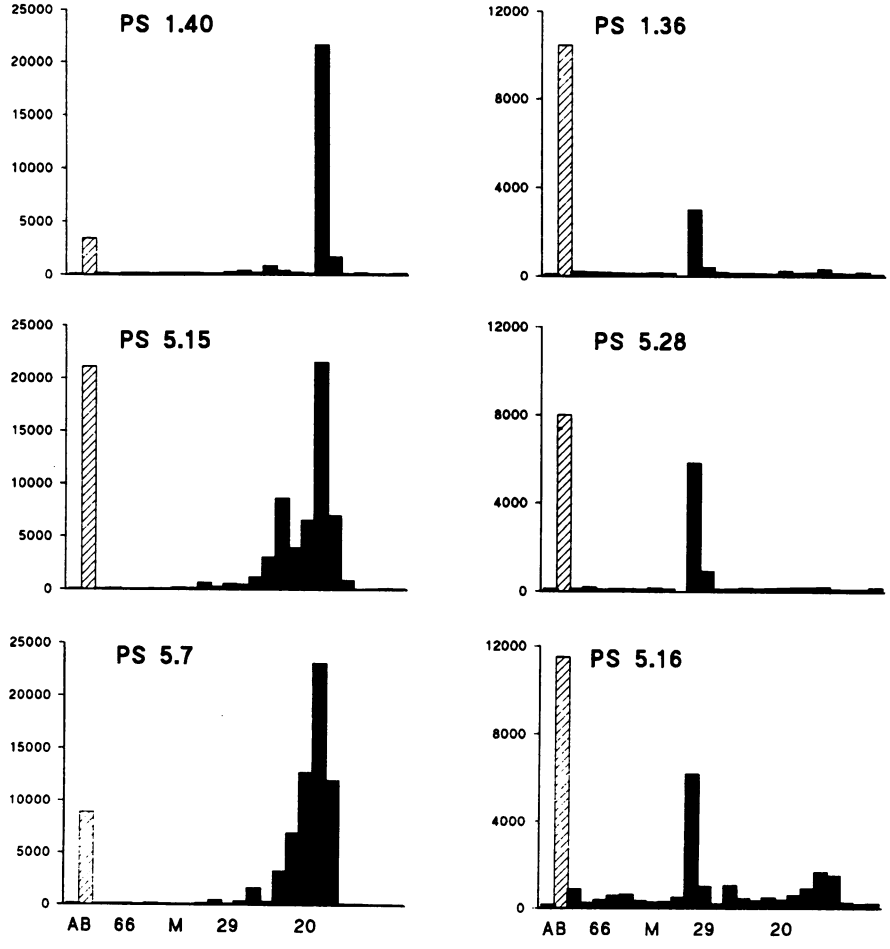

Figure 5. Proliferative responses of 4×10^4 clone T cells of each of six clones to each of 24 strips of nitrocellulose blotted with PAGE-separated chlamydial proteins. The sizes of MW markers are shown on the *x*-axes ($\times 10^3$). Bar A represents the response to nitrocellulose spotted with PBS, while bar B (■) represents the response to purified strain DK20 EB. M represents the fraction in which the major outer membrane protein would be expected to be found. Data are shown from four experiments.

In this study, 61 chlamydia-specific clones were isolated from a single cloning of which six were characterized in detail, and the antigen specificity of a further four also determined. All were $CD4^+$, which is in keeping with our observations of polyclonal synovial fluid T-cell responses to reactive arthritis-associated organisms which are dominated by MHC class II-restricted $CD4^+$ T cells.[12] HLA-B27-restricted pathogen-specific responses have not been identified in reactive arthritis, despite using strategies aimed at demonstrating such responses.[24] The precise MHC restriction of these clones proved interesting. All six clones were DR1 restricted; three were extremely sensitive to the polymorphism at positions 85/86 on the DR β-chain in that they could only respond to chlamydial antigen presented by $DRB1*0101^+$ cells, but not by $DRB1*0102^+$ cells which differ from the former only at these two residues. The identical fine specificity of MHC restriction shown by these clones was suggestive that they were recognizing the same antigen, as shown by the results of T-cell immunoblotting. It is notable that Ong *et al.* reported DR4-restricted acetyl choline receptor-specific clones which were similarly sensitive to the polymorphism at position 86.[25] These authors speculate that the flexibility of glycine 86 may permit a conformational state which cannot be achieved with other residues at this site. It is not clear whether the sensitivity of T cells to changes at this site is a result of effects (direct or indirect) on peptide binding, or of a direct effect on recognition of the peptide/DR molecule complex by the T-cell receptor. Busch *et al.* have recently described the polymorphism at residue 86 as having a major effect on peptide binding.[26]

The T-cell immunoblotting technique allowed the identification of two distinct chlamydial antigenic fractions, one of 18,000 MW and the other 30,000 MW. The technique proved consistent and highly reproducible. It is interesting that nine clones, chosen from 61 produced, should between them recognize only two antigens. It is not yet clear whether these clones represent *in vitro*-or *in vivo*-derived sister clones. We are addressing this question by investigating their T-cell receptor usage. We are also assessing recognition of these antigens by the remaining clones, since testing the uncloned SFMC by immunoblotting suggested that additional chlamydia antigens were eliciting T-cell responses. Both the antigens identified appear quite separate from the major outer membrane protein (MOMP) of chlamydia to which a significant proportion of antibody responses is known to be directed.[15] The MOMP was clearly apparent as a characteristic band on gel electrophoresis of solubilized preparations of strain DK20, and also on stained preparations of the blotted nitrocellulose used in the clone assays. T-cell responses to MOMP have been described and characterized in the mouse following immunization with the protein.[27] Since immunoblotting showed that the patient had antibodies specific for MOMP, it is likely that MOMP-specific T cells were also present in the patient providing specific help for the antibody response, but it is of interest that none of the clones was shown to recognize this antigen. It cannot be excluded that the clone for which no specificity was demonstrated by T-cell immunoblotting actually responded to MOMP but could not recognize the antigen bound to nitrocellulose.

As yet the importance of MOMP-specific T-cell responses in SARA is unknown. Other candidate T-cell chlamydial antigens include the 60,000 MW cysteine-rich protein OMP2,[28] and the 57,000 MW stress protein of chlamydia.[29] The presence of antibodies to the 57,000 MW protein has been reported as being more prevalent in women with serious post-salpingitis sequelae than in those without such sequelae,[30] and has been associated with tubal infertility.[31] Elevated levels of antibody to a 60,000 MW chlamydial protein have also been observed in individuals with SARA.[32] However, no significant clonal response to antigens of 60,000 MW was detectable in this study. In theory, one or both of the fractions recognized could represent breakdown products of a 60,000 MW protein. However, solubilization of the chlamydial antigens, and preparation of the blots, were performed in the presence of the protease inhibitor phenyl methyl sulphonyl fluoride to prevent common protease-mediated breakdown.

We have not yet defined the exact nature of the chlamydial antigens identified. We are currently extending this work with the aim of isolating the antigen as an homogeneous polypeptide whose N terminal sequence can then be determined. Our strategy towards this end includes the use of two-dimensional separation of proteins and subsequent T-cell immunoblotting. We are also using eluates of the antigenic bands which can then be further purified by techniques such as high performance liquid chromatography.

The definition of chlamydial antigens recognized by T cells, as yet in its infancy, remains of great importance for at least two reasons. Firstly, such characterization will facilitate a greater understanding of the pathogenesis of chlamydial diseases. Secondly, such antigens may have a therapeutic role, for example in designing vaccines. Pertinent to this was the finding during initial vaccine trials that in some cases, the incidence of trachoma was actually higher in those children who had received the chlamydial vaccine than in those who had not,[33] lending support to the delayed-type hypersensitivity experimental model of disease. The aim would be to design a vaccine which avoids antigens which provoke hypersensitivity responses, but includes those antigens affording protection by antibodies. The approach taken in this study has allowed initial identification of two antigens recognized by T cells. Extension of this work will allow further characterization of these antigens as well as identification of others.

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