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Preparative separation of foreign antigens for highly efficient presentation to T cells in vitro

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A method is described for the separation and purification of proteins from complex mixtures of foreign antigens in a form suitable for stimulating T cells in vitro. The technique involves electrophoretic separation of proteins followed by elution, concentration and adsorption of the polypeptide subunits to latex microspheres. Alternatively, where a specific antibody is available, proteins may be affinity-purified from a heterogeneous mixture of antigens, using antibody-coated latex microspheres. Nanogram quantities of protein coupled to latex were shown to be highly efficient stimulators of antigen-specific T cells as tested by in vitro proliferation and cytokine release assays. The utility of this technique was demonstrated using poliovirus capsid proteins separated by SDS-polyacrylamide gel electrophoresis (PAGE) and coupled to latex microspheres for specificity analysis of T cell clones. Antigen reactivity of the T cell clones was confirmed using recombinant baculoviruses expressing individual poliovirus proteins. Furthermore, recombinant proteins coupled to latex microspheres were used for efficient stimulation and in vitro propagation of T cell clones specific for the simian immunodeficiency virus (SIV) envelope (env) protein. Although the technique is illustrated in this report using viral antigens, it has also proved to be an efficient method for the separation of bacterial antigens in studies of polyclonal T cell responses to *Bordetella pertussis* antigens.

Key words: Viral antigen; Bacterial antigen; T cell proliferation; T cell clone; Latex microsphere; SDS-PAGE

Introduction

An increased understanding of the immune recognition of foreign antigens by T cells and the mechanisms of protective immunity to infectious pathogens is dependent on simple and reproducible methods for the assessment of T cell responses. Unlike antibodies which bind to conformational epitopes directly, T cells recognise antigens presented as linear epitopes in association with MHC molecules following processing by antigen presenting cells (APC) (Mills, 1986; Allen, 1987; Vitetta et al., 1989). The recognition of short linear sequences by antigen-specific T cells makes it possible to identify T cell epitopes using synthetic peptides corresponding to the epitope in the native antigen. However, suitable prepara-

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Abbreviations: PAGE, polyacrylamide gel electrophoresis; SIV, simian immunodeficiency virus; env, envelope; APC, antigen-presenting cells; PBS, phosphate-buffered saline; FCS, foetal calf serum.

tions of purified protein components of viruses and bacteria are essential for the first phase of antigen specificity analysis.

A number of approaches including gel chromatography techniques and a variety of recombinant expression systems have been used for the preparation of viral and bacterial subunits (Mackett, 1987; Henry, 1989). However, cloned genes for a range of protein components especially for complex pathogens such as bacteria and parasites are rarely available. Furthermore, existing separation techniques for native antigens are technically elaborate (Heukshoven and Dernick, 1985) or require relatively large amounts of purified antigen, a particular problem with viruses which are difficult to propagate and purify. An alternative approach has been the use of nitrocellulose-bound antigen blotted from polyacrylamide gel-separated viral or bacterial lysates in proliferation assays either directly or as a particulate suspension after dissolution in dimethylsulphoxide (Young and Lamb, 1986; Abou-Zeid et al., 1987; Hutchings et al., 1990). However, we and others (Guttinger et al., 1989; Hutchings et al., 1990) found this technique irreproducible and not very sensitive.

In this report we describe an alternative technique which overcomes many of the problems with the so-called T cell Western technique. Disrupted viral proteins were electrophoresed on SDS-polyacrylamide gels and identified protein bands eluted, concentrated and adsorbed onto latex microspheres. Such preparations stimulated polyclonal and cloned T cells in a highly efficient manner. We have applied the technique to the separation of poliovirus capsid proteins for specificity analysis of murine poliovirus-specific $CD4^+$ T cell clones. In addition, recombinant proteins coupled to latex beads have been successfully used for the in vitro propagation of macaque T cell clones specific for the SIV env protein.

Materials and methods

Antigens

Poliovirus Sabin types 1, 2 and 3 vaccine strains were propagated and titrated in Hep2c cells according to established protocols (Minor, 1985). Virus from infected tissue culture fluid was concentrated by the addition of 0.4 g/ml ammonium sulphate and the mixture centrifuged at $6000 \times g$ for 4 h at 4°C. The resultant pellet was resuspended in phosphate-buffered saline (PBS) and purified through a 15–45% sucrose gradient. Fractions containing virus were pooled, pelleted by centrifugation at $30{,}000 \times g$ for 16 h and resuspended in PBS; typically, the final concentrate contained 10^{10} PFU/ml of infectious virus.

The coding regions of poliovirus type 3 capsid proteins VP0 (VP2 + VP4), VP1 and VP3 were used to prepare recombinant baculoviruses based on *Autographa californica* nuclear polyhedrosis virus. The cDNA sequences encoding the three capsid proteins were cloned and expressed individually under the control of the polyhedrin promoter, as described by Bräutigam et al. (1992). Expression of these proteins was performed in infected *Spodoptera frugiperda* cells.

The gp140 protein of SIV expressed in baculovirus, purchased from Repligen as a purified protein in 1 M urea and 0.05% SDS, was provided by the UK MRC AIDS Directed Programme.

Separation of poliovirus capsid proteins

Poliovirus capsid proteins were resolved on SDS-PAGE using a discontinuous buffer system as described by Laemmli (1970). Viral concentrates were boiled for 5 min in disruption buffer (0.2%-SDS, 0.5% β-mercaptoethanol in 0.1 M Tris buffer, pH 6.8) and loaded onto gels containing 15% acrylamide cross-linked with 0.14% bisacrylamide. After electrophoresis for 18 h at 7 mA, vertical strips were excised from the edges of the gel and silver stained. The protein bands corresponding to VP1, VP2 and VP3 on the remaining unstained part of the gel were identified by alignment with the silver-stained gel strips. As the VP4 protein of type 3 poliovirus is difficult to locate by silver staining, rainbow markers (Amersham International) that included 14.3 kDa and 6.5 kDa molecular weight coloured proteins were used to identify and remove the gel band in the region of 7 kDa known to contain VP4. Individual gel slices containing virus proteins were placed in physiological PBS diluted to 50% with water (0.5 ml per gel band), incubated for 30 min

at 35°C and then overnight at 4°C. The bulk of the SDS was removed by placing the eluates in crushed ice for 4 h, and the crystallised SDS removed by pelleting in a bench Microfuge. Proteins were concentrated through Centricon-10 tubes (Amicon) and resuspended in PBS prior to storage at −70°C.

Coupling of proteins to latex microspheres

Gel-eluted poliovirus capsid proteins or recombinant SIV env protein or disrupted virus polypeptides were mixed with 100 μl of a 10% (w/v) suspension of latex microspheres (0.8 μl diameter, Sigma) in 0.1 M glycine-NaOH buffer, pH 8.6. The mixture was incubated at 4°C with continuous agitation for 16–18 h. After pelleting at 13,000 × *g* in a bench microfuge, unbound sites were blocked by mixing the microspheres with PBS containing 10% foetal calf serum (FCS) for a further 6 h at 4°C. After repelleting in a microfuge, microspheres were washed twice in PBS with 2% FCS, and sterilized by placing in a petri dish and exposing to a UV light source at a distance of 8 cm for 10 min. The latex microspheres were resuspended to the original concentration in RPMI culture medium containing 8% FCS, and stored at 4°C for 1 week or at −70°C for an indefinite period.

Affinity purification of poliovirus VP1 for use in T cell assays

Rabbit antiserum (R78) raised against a synthetic peptide corresponding to residues 89–100 of VP1 of poliovirus type 3 was donated by Morag Ferguson and prepared as described in Ferguson et al. (1985). Purified IgG (100 μl, 2 mg/ml) prepared from R78 was mixed with 100 μl of 10% (w/v) suspension of latex microspheres in 0.1 M glycine-NaOH buffer pH 8.6 followed by continuous agitation for 16 h at 4°C in the same buffer. After blocking the unbound sites with PBS containing 10% FCS, 100 μl of SDS-disrupted type 3 virus polypeptides were added and the mixture incubated for a further 16 h at 4°C.

Finally, latex microspheres were washed in PBS, UV sterilized as described above and resuspended to the original concentration in RPMI containing 8% FCS prior to storage.

Generation of poliovirus-specific T cell clones

Murine $CD4^+$ T cell clones specific for Sabin types 1, 2 and 3 were established as described by Mahon et al. (1992). Briefly, spleens were removed from BALB/c ($H\text{-}2^d$) and CBA ($H\text{-}2^k$) mice 14 days after immunization with approximately 10^8 pfu of purified poliovirus. T cell lines were generated by in vitro stimulation of spleen cells with purified poliovirus (2×10^7 pfu), followed by addition of fresh medium and feeder cells after 5 days. Established lines were cloned by limiting dilution and maintained by re-stimulation with antigen and APC (syngeneic irradiated spleen cells at 2×10^6/ml), every 10 days, with IL-2, feeder cells and fresh medium added 5 days after antigen stimulation.

Generation of SIV env-specific T cell clones

$CD4^+$ T cell clones specific for the SIV env protein were established from macaques immunized with recombinant env proteins as described by Jones et al. (1992). Briefly, PBMC from immunized macaques were stimulated at 5×10^5/ml with Baculovirus-expressed SIV gp140 adsorbed to latex microspheres at a nominal antigen concentration of 0.1 μg/ml, assuming 100% protein binding to the microspheres. T cell clones were generated from T cell lines by limiting dilution and maintained by re-stimulation with latex-coupled gp140 and autologous APC (irradiated PBMC, 5×10^5/ml) every 14 days, with the addition of IL-2, 5 days after antigen stimulation.

Proliferation and IL-2 assays

T cell clones (1×10^5/ml) were cultured with antigen and APC (irradiated autologous PBMC, 5×10^5/ml for macaque T cells and syngeneic spleen cells, 2×10^6/ml for murine cells) for 3 days in flat bottomed microtiter plates. Native or disrupted virus, synthetic peptides or antigen coupled to latex microspheres (10% w/v) were serially diluted (five-fold) and 25 μl added to 200 μl of cells. Cultures were pulsed with [^{3}H]thymidine for 4 h and incorporated label counted by liquid scintillation. Results were expressed as mean cpm of [^{3}H]thymidine incorporation for triplicate cultures.

IL-2 production by stimulated T cells was assessed in identical assays by testing the ability of

supernatants to support the growth of an IL-2 dependent CTLL cell line as previously described (Katrak et al., 1991).

Results

Highly efficient stimulation of T cells by viral proteins coupled to latex microspheres

Detergent disrupted poliovirus proteins coupled to latex microspheres were highly efficient stimulators of in vitro proliferative activity of antigen-specific T cells. Initial experiments showed that denatured virus proteins induced higher levels of T cell proliferation when compared with a similar concentration of intact virus particles (data not shown). However, when SDS-disrupted virus proteins were coupled to latex microspheres, a significant enhancement of T cell proliferative activity was detected compared with that obtained using a similar concentration of uncoupled native or disrupted virus (Fig. 1 and data not shown). For example, the proliferative response of the poliovirus-specific murine $CD4^+$ T cell clone 3N2s5.1 was measured at approximately 40,000 cpm for the highest concentration of disrupted virus coupled to latex microspheres, as opposed to 12,000 cpm for the uncoupled preparation (Fig. 1). This response was antigen-specific and dose-dependent; FCS-coupled latex microspheres did not stimulate T cell proliferation. The lowest concentration of virus protein coupled to latex microspheres capable of inducing a positive proliferative response was calculated to be approximately 5 ng (assuming 100% binding) whereas 500 ng of uncoupled protein was required to stimulate comparable levels of proliferation. FCS-coupled latex microspheres did not stimulate T cell proliferation (Fig. 1). Furthermore the addition of FCS-coupled latex microspheres did not enhance the response to the uncoupled disrupted virus (data not shown).

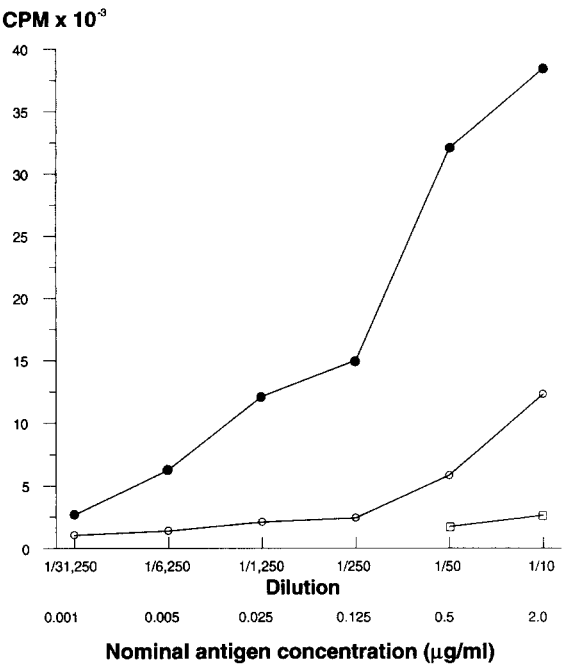

Fig. 1. Proliferative response of poliovirus-specific T cell clone 3N2s5.1 to disrupted type 3 poliovirus before (○) and after (●) coupling to latex microspheres and to FCS-coupled latex microspheres (□). The nominal antigen concentration shown on the *x* axis represents the estimated final concentration of uncoupled disrupted virus protein and latex microsphere-adsorped disrupted virus protein (assuming 100% binding).

Specificity analysis of poliovirus-specific T cell clones using gel-separated capsid proteins coupled to latex microspheres

Poliovirus capsid proteins VP1, VP2, VP3 and VP4 eluted from gel slices after electrophoresis, concentrated through Amicon centrifuge tubes and coupled to latex microspheres, were prepared as antigens for use in T cell assays. Prior to coupling to latex microspheres, protein eluates were re-electrophoresed alongside parental virus to check the purity of each preparation. The result clearly established the homogeneity of each viral capsid protein with no detectable cross-contamination (Fig. 2). Gel-separated latex microsphere-coupled proteins were used for specificity analysis of a panel of poliovirus-specific $CD4^+$ T cell clones. Table I shows representative data for five T cell clones, four of which map to distinct viral capsid proteins; T cell clones 3N2s5.1, 1N8.44, CB2.2-10 and 2KA-2 responded specifically to VP1, VP2, VP3 and VP4 respectively. Clone 2KB-3 which was specific for type 2 poliovirus, did not recognize any of the purified capsid proteins prepared from type 3 poliovirus. Clonal specificity was confirmed using recombinant baculovirus constructs expressing poliovirus

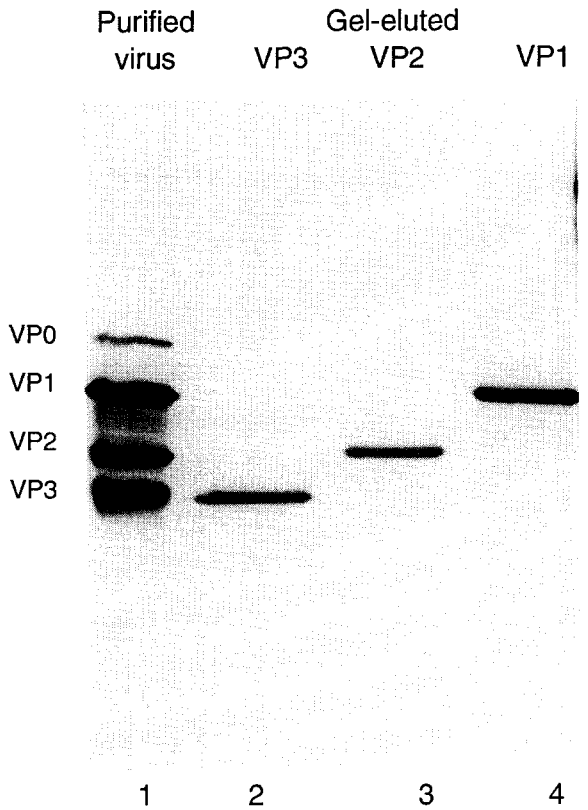

Fig. 2. Purity of separated poliovirus capsid proteins. SDS-PAGE of proteins eluted from excised gel bands and concentrated through Centricon tubes (lanes 2–4). Lane 1 shows the typical migration pattern of a poliovirus concentrate.

type 3 VP0 (VP2 + VP4), VP1 and VP3 sequences.

T cell stimulation with affinity purified poliovirus VP1 coupled to latex microspheres

As latex microspheres were capable of binding to a heterogeneous mixture of SDS-disrupted viral proteins, we investigated the ability of immune IgG coupled to latex microspheres to purify a specific virus protein. Purified IgG prepared from the serum of a rabbit hyper-immunized with a synthetic peptide containing a poliovirus VP1 sequence was coupled to latex microspheres and mixed with a lysate of SDS-disrupted poliovirus proteins. The results demonstrated that the VP1-specific T cell clone 3N2s5.1 responded in an antigen-specific dose-dependent manner to the captured VP1; no activity was shown by immune IgG bound to latex microspheres or by latex microspheres blocked with FCS, incubated with poliovirus lysate and washed free of unbound proteins (Fig. 3). Furthermore, the latex-IgG-VP1 preparation failed to stimulate T cell clones specific for VP2, VP3 and VP4 (data not shown). This result confirmed the specificity of T cell clone 3N2s5.1 for VP1 as shown in Table I and illustrated the use of affinity-purified proteins for T cell specificity analysis.

TABLE I

SPECIFICITY OF T CELL CLONES FOR DISTINCT POLIOVIRUS CAPSID PROTEINS

T cell clone	Medium	Whole virus [b]	Proliferative response [a] to						
			Gel separated and latex microsphere coupled				Baculovirus expressed		
			VP1	VP2	VP3	VP4	VP1	VP0 [c]	VP3
3N2s5.1	94	9,927 [d]	9,600	232	270	40	5,300	n.d.	n.d
1N8.44	62	7,983	61	3,942	90	n.d.	n.d.	3,500	n.d.
CB2.2-10	1,416	92,355	1,506	1,669	90,172	1,310	2,291	1,455	10,345
2KA-2	424	41,422	720	530	630	90,400	398	16,574	514
2KB-3	710	9,450	640	760	527	672	1620	1,200	801

[a] Results represent optimum response against a range of antigen dilutions.
[b] Response to poliovirus type 3, except for clone 2KB-3 which is type 2 specific; the response for this clone is shown for type 2 poliovirus.
[c] VP0 is the precursor of VP2 and VP4.
[d] Responses considered positive are underlined.

Generation and maintenance of T cell clones using viral antigens coupled to latex microspheres

In an attempt to study the T cell recognition of SIV proteins, commercially available SIV env protein at 50 μg/ml in 0.05% SDS and 1 M urea was adsorbed to latex microspheres and used to stimulate SIV env-specific proliferative T cell responses in PBMC from immunized macaques (Mills et al., 1991 and data not shown). This antigen preparation was then used for the propagation of T cells in vitro; panels of T cell lines and clones specific for the SIV env proteins have been successfully established and maintained using latex-coupled SIV env protein at a nominal concentration of 0.1 μg/ml (Jones et al., 1992 and data not shown). A typical dose-response curve of an SIV env-specific T cell clone against a range of dilutions of latex-coupled antigen is shown in Fig. 4. This T cell clone responds to a synthetic peptide corresponding to residues 321–340 but not to the adjacent peptide 341–360. In comparison with peptide, the latex microsphere adsorbed SIV env protein induced significantly higher levels of proliferation and IL-2 production

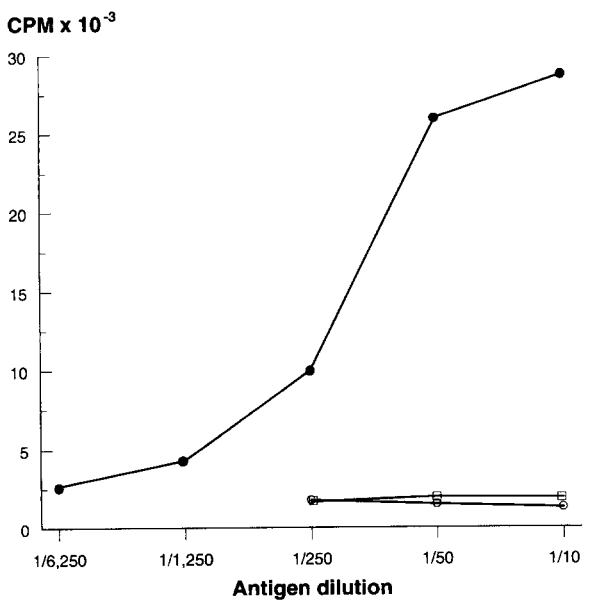

Fig. 3. Proliferation response of poliovirus-specific T cell clone 3N2s5.1 to VP1 purified from viral lysates on anti-VP1 IgG-coated latex microspheres (●); to IgG-coated latex microspheres alone (○) and blocked latex microspheres incubated with and washed free of disrupted virus (□).

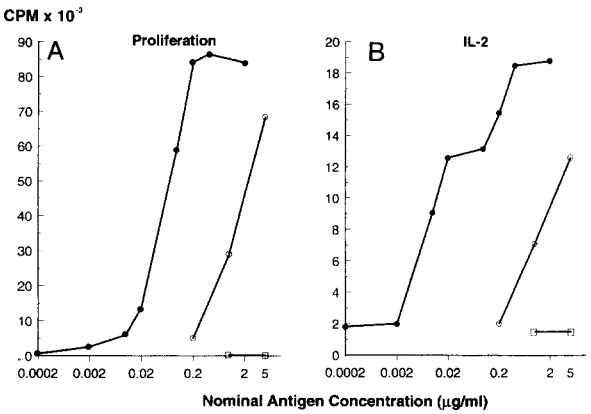

Fig. 4. Proliferation (*A*) and IL-2 (*B*) responses of a macaque $CD4^+$ T cell clone specific for peptide 321–340 of the SIV env protein. Responses were tested against SIV env coupled latex microspheres (●); peptide 321–340 (○) and peptide 341–360 (□). Estimated antigen concentration in culture assumes 100% binding of gp140 to latex microspheres. Results are shown as $[^3H]$thymidine incorporation for triplicate cultures. IL-2 was detected by CTLL proliferation.

by the T cell clone at lower antigen concentrations. Positive responses could be detected against the env protein coupled to latex microspheres at concentrations as low as 3–10 ng/ml. At the coupling concentration used, greater than 90% of the protein was found to be adsorbed to the latex microspheres.

Discussion

In this report, we describe a method for the separation of protein subunits from complex mixtures of antigens for efficient stimulation of T cells in vitro. In our technique, viral or bacterial lysates were electrophoresed on conventional SDS gels and proteins eluted from identified gel bands by simple incubation in hypotonic PBS. After concentration, proteins were adsorbed to latex microspheres and washed free of SDS. Foreign antigens purified in this way were capable of inducing consistent antigen-specific proliferation and lymphokine secretion in both cloned and polyclonal T cells over a range of dilutions. Apart from its simplicity and efficiency, this technique permits an assessment of the composition of antigen fractions, titration of the dose of antigen

added to the T cells and repetition of a large number of assays using material from a single preparation.

Our technique was first developed for studies on T cell recognition of poliovirus using a panel of antigen-specific $CD4^+$ T cell clones. Prior to the identification of T cell epitopes with sets of overlapping synthetic peptides, we sought to establish their specificity for viral capsid proteins VP1, VP2, VP3 and VP4. As relatively large amounts of protein are required for purification by methods such as HPLC (Heukeshoven and Dernick, 1985), we investigated alternative methods for the extraction and purification of viral capsid proteins (Abou-Zeid et al., 1987; Young and Lamb, 1986). Attempts to stimulate T cell clones in in vitro assays with nitrocellulose bound capsid proteins blotted from SDS gels either directly or as a particulate suspension were unsuccessful. Therefore we examined methods of improving the T cell stimulatory capacity of gel-separated fractions. We had already observed that disrupted poliovirus or recombinant SIV proteins adsorbed to latex microspheres significantly increased their antigenicity for T cells. The improved antigen presentation and T cell stimulation may result from enhanced APC uptake of the antigen in particulate form. When applied to the purification of poliovirus proteins from gel eluates, this technique produced fractions of high purity and permitted the identification of distinct capsid proteins recognised by a panel of poliovirus-specific T cell clones. Following the development of the technique, baculovirus expressed poliovirus proteins VP0 (precursor of VP2 and VP4), VP1 and VP3 became available (Bräutigam et al., 1992). The pattern of responses of the T cell clones against the recombinant proteins confirmed the specificity established with latex microsphere-adsorbed material separated from viral lysates.

An alternative approach for the purification of poliovirus VP1 involved the coupling of a specific antibody to latex microspheres for affinity purification. A polyclonal rabbit IgG specific for VP1 was adsorbed to latex and used to affinity-purify VP1 from a mixture of disrupted virus proteins. This preparation stimulated the proliferative response of a T cell clone in a dose-dependent manner, confirming the specificity of this clone for VP1, as well as the antigenicity of affinity-purified VP1. Although our technique was developed and is illustrated in this report for viral antigens, it also has considerable potential for studies of T cell recognition of bacterial and parasitic antigens. Due to the large number of subunits on these pathogens, specific antibodies or recombinant proteins are unlikely to be available for the complete array of proteins. Indeed we have recently used latex microsphere-adsorbed SDS-PAGE separated fractions from a *B. pertussis* lysate for specificity analysis of polyclonal T cells specific for *B. pertussis*. (Barnard, Redhead and Mills, unpublished data).

The technique described in this report is not only an efficient technique for the preparative separation of antigens for use in T cell assays, but is also a valuable method for presentation of antigens in the propagation of antigen-specific T cell lines and clones. SIV env-specific T cell clones were established from immunized macaques and maintained in culture by repeated stimulation with autologous APC and antigen coupled to latex microspheres. The antigen used was baculovirus expressed gp140 obtained from commercial sources at a concentration of 50 μg/ml in 1 M urea and 0.05% SDS and was only available in small quantities due to cost (GBP1000/mg). Adsorption to latex microspheres allowed us to remove the detergent and urea, both toxic to cells in culture, and to use the protein in extremely low concentrations for re-stimulation of T cells. Panels of SIV env-specific T cell lines and clones have been successfully generated in our laboratories using this approach (Jones et al., 1992).

In conclusion, our findings demonstrate that elution of protein bands from SDS-PAGE followed by adsorption to latex microspheres is a reliable and sensitive technique for the separation of proteins from complex mixtures of antigens for presentation to T cells. The technique can be used for the identification of distinct protein subunits recognised by T cells generated against the intact foreign pathogen. Furthermore it can be applied to the propagation of T cell clones against antigens separated by SDS-PAGE. The application of this technique may be particularly useful in studies of T cell recognition of

complex microorganisms or pathogens such as retroviruses and hepatitis virus where there are difficulties in their propagation and purification.

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