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In vitro stimulation of peripheral blood mononuclear cells (PBMC) from HIV$^-$ and HIV$^+$ chancroid patients by *Haemophilus ducreyi* antigens

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SUMMARY

The cellular immune responses to fractionated *Haemophilus ducreyi* antigens, coated on latex beads, were assessed in patients with chancroid and in controls, using an *in vitro* lymphocyte proliferation assay. Several fractions of *H. ducreyi* antigen revealed stimulating activity. However, only the molecular size ranges 91–78 kD, 59–29 kD, and 25–21 kD induced proliferation that may be specifically related to *H. ducreyi* infection. Lymphocytes from four HIV$^-$ patients, successfully treated for chancroid, were not stimulated by *H. ducreyi* antigen. In general, lymphocytes from HIV$^+$ chancroid patients were less responsive to *H. ducreyi* antigen compared with those from HIV$^-$ chancroid patients. However, two HIV-infected patients showed exceptionally strong responses to high molecular weight fractions. To our knowledge this is the first report demonstrating that *H. ducreyi* contains specific T cell-stimulating antigens. Based on this work, further identification and purification of the T cell antigens is feasible.

Keywords chancroid *Haemophilus ducreyi* cellular immune response HIV

INTRODUCTION

Chancroid is the major cause of genital ulcer disease in developing countries, and a well documented risk factor for the heterosexual transmission of HIV [1,2].

Haemophilus ducreyi, the causative agent of chancroid, is a Gram-negative human pathogen. Various animal models for chancroid were developed [3,4]. Thus, chancroidal ulcers were induced in a temperature-dependent rabbit model [3] as well as in a primate model [4], but the relevance of these models for the pathogenesis and cellular immune responses in human chancroid remains to be established. Yet, little is known about the pathogenesis of *H. ducreyi* infection in humans. *In vitro* cell culture methods (i) have demonstrated that *H. ducreyi* produces a cytotoxin which specifically causes death of human epithelial cells [5], and (ii) have provided evidence for *H. ducreyi*-lipopolysaccharide (LPS)-associated tissue necrosis [6]. Surprisingly, controversy persists on the question whether *H. ducreyi* is an intra- or extracellular pathogen [7–9].

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Very few data are available on the human immune response to *H. ducreyi*. Biopsies taken from the lesions showed the presence of neutrophils and numerous eosinophils [10]. The purulent nature of the disease may support the involvement of polymorphonuclear cells in the defence against *H. ducreyi*. Odumera *et al.* [11] observed that avirulent strains display a higher susceptibility to the complement-mediated bactericidal activity of normal human serum and to phagocytosis and killing by human polymorphonuclear cells. The LPS composition may contribute to this susceptibility. Besides polymorphonuclear cells, macrophages and T cells were observed in biopsies [12]. The involvement of T cells in the immune response against *H. ducreyi* is indirectly suggested by (i) a delayed hypersensitivity reaction observed in many patients with severe chancroid, (ii) the less favourable clinical evolution of chancroid in HIV$^+$ individuals, and (iii) the presence of lymphadenopathy in HIV$^-$ chancroid patients as well as the absence of this symptom in most HIV-infected chancroid patients [13,14].

According to Spinola *et al.* [12] B cells were absent in the mononuclear infiltrates seen in biopsies, but the demonstration of anti-*H. ducreyi* serum antibody proves that humans mount a humoral response [15–17]. Preliminary analysis of the kinetics of antibody synthesis in humans revealed a more complex pattern than described in other bacterial infections [18].

Table 1. Subject groups

Chancroid patients (Nairobi)			
HIV$^-$ *Hd*cult.$^+$	HIV$^-$ *Hd*cult.$^-$	HIV$^+$ *Hd*cult.$^+$	*HIV*$^+$ *Hd*cult.$^-$
10*	1	3	4
Control individuals			
HIV$^-$ RPR$^+$ (Nairobi)	HIV$^-$ (Antwerp)	HIV$^+$ (Antwerp)	
2	15	5	

*Six untreated, four treated.
RPR, Rapid Plasma Reagin test.

Furthermore, these antibodies display a high cross-reactivity with antigens from related species [19].

Yet the benefits to the infected host provided by these various immune responses are not very clear. Complete protective immunity is apparently non-existent, since reinfection is common and repeated autoinoculations were observed in early studies of chancroid [20]. To understand better the role of the immune response in the interaction between host and agent, the present study assessed *in vitro* lymphoproliferative responses in chancroid patients to identify important *H. ducreyi* immunogenic determinants.

SUBJECTS AND METHODS

Subjects

After informed consent 11 HIV$^-$ and seven HIV$^+$ male Africans, who presented at the Special Treatment Clinic (STC) in Nairobi (Kenya) with genital ulcer disease (GUD), were selected for the study. In those patients the clinical diagnosis of chancroid was confirmed by culture in 13 cases. Of the five culture-negative patients four were HIV$^+$ and one was HIV$^-$. They showed high optical density (OD) ratios in an experimental enzyme immunoassay (EIA) [17]. Four of the 11 HIV$^-$ patients were seen 2–4 months after culture-confirmed diagnosis and successful treatment with erythromycin (250 mg, three times a day for 7 days; Orbi-Pharma, Antwerp, Belgium). They returned to the STC because they were enrolled in a longitudinal study in GUD.

A first group of negative controls consisted of two *H. ducreyi*-culture-negative patients with genital ulcers. They were seronegative for HIV and *H. ducreyi* antibodies, but positive for syphilis in the Rapid Plasma Reagin test (RPR; Becton Dickinson, Cockeysville, MD). Fifteen healthy volunteers working at the Institute of Tropical Medicine were selected as negative controls for the HIV$^-$ patient population. They included three individuals of African origin, 11 of Caucasian origin and one of Asian origin. Five HIV$^+$ patients (one African, three Caucasians and one Asian) presenting at the out-patients clinic of the Institute of Tropical Medicine were used as negative controls for the HIV$^+$ chancroid patients (Table 1).

Sample handling

From each individual, 10 ml of blood were collected in EDTA (Sarstedt, Nümbrecht, Germany) by venous puncture. PBMC were isolated by Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation, washed and frozen in 50% (v/v) heat-inactivated autologous serum, 10% (v/v) dimethylsulfoxide (Merck, Darmstadt, Germany) and 40% (v/v) RPMI (Gibco-BRL, Gaithersburg, MD) using the Cryo 1°C freezing container (Nalgene, Rochester, NY), as described previously [21]. The frozen PBMC were stored and transported in liquid nitrogen.

Antigen preparation

Crude *H. ducreyi* cocktail antigen (600 μg of protein) was fractionated using preparative SDS–PAGE (Gibco-BRL) as described elsewhere [22]. To allow proper determination of the molecular size of the proteins, the protein pattern was visualized using reversible staining with $CuCl_2.2H_2O$ (Merck) [23], before the gel was cut into 20 equally sized pieces. Elution of proteins out of the gel slices and binding of the eluted proteins to latex beads were performed according to Katrak *et al.* [24] with minor modifications. Briefly, gel slices were destained and covered with two-fold diluted PBS (pH 7·4; 1·5 mM KH_2PO_4, 8·1 mM Na_2HPO_4, 2·7 mM KCl, 140 mM NaCl) (Merck). Subsequently, they were placed at 30°C for 30 min, and at 4°C overnight. To remove excess SDS, eluates were incubated on crushed ice for 4 h and were centrifuged at 4°C in a bench Microfuge (Heraeus Sepatech, Hanau, Germany). Eluates were concentrated by centrifugation using Centricon-10 (Amicon, Beverly, MA). The amounts of protein (Pierce BCA Protein Assay; Pierce, Rockford, IL) eluted from the different gel slices and coated to the latex beads, as well as the resulting molecular size ranges of the different fractions, are shown in Table 2.

The concentrated protein fractions (100 μl) were coupled to 100 μl of a 10% (w/v) suspension of latex beads (0·8 μm diameter; Sigma, St Louis, MO) by overnight incubation at 4°C in 0·1 M glycine–NaOH (Gibco-BRL), pH 8·6. The coated latex beads were pelleted and the binding efficiency of the protein to the latex beads was assessed. Binding appeared to be complete as protein was no longer determined in the supernatant. Unbound sites were blocked by incubation for 6 h at 4°C with 10% (v/v) human AB + serum (Advanced Protein Products, West Bromwich, UK) in PBS. Beads were washed twice in PBS containing 2% (v/v) human AB + serum and were resuspended in RPMI containing 8% (v/v) human AB + serum. This suspension was sterilized under a UV light source (302 nm), aliquoted and stored at −70°C. Negative control latex

Table 2. Amount and molecular size range of the various *Haemophilus ducreyi* antigen fractions

Fraction number	Eluted protein (μg)	Molecular size range (kD)
1	4·4	>91–91
2	5·4	91–78
3	12·4	78–67
4	15·2	67–59
5	19·7	59–51
6	8·9	51–44
7	10·9	44–38
8	19·1	38–33
9	13·1	33–29
10	7·0	29–25
11	3·1	25–21
12	4·0	21–19
13	10·6	19–16
14	15·9	16–14
15	9·7	14–12
16	4·6	12–11
17	4·0	11–9
18	1·7	9–8
19	1·3	8–7
20	0·9	7–6

beads were treated as described above, but in the absence of *H. ducreyi* antigen.

Proliferation assay

Cryopreserved PBMC were rapidly thawed at 37°C and washed twice in RPMI containing 10% (v/v) fetal calf serum (FCS; GIBCO-BRL). Subsequently, the PBMC were resuspended in RPMI containing 20% (v/v) human AB + serum at a density of 10^6 cells/ml. The cells were aliquoted in U-bottomed 96-well microtitre plates (100 μl/well). Latex beads were diluted 200-fold in RPMI and sterilized. Subsequently, 100 μl of the suspension were added to the PBMC. Depending on the amount of PBMC recovered from each patient, proliferation was measured in duplicate, triplicate or quadriplate. Soluble tetanus toxin (TT; Institut Mérieux Benelux N.V., Brussels, Belgium) or Varicella/Zoster virus antigen (VZ; Behring, Marburg, Germany), and phytohaemagglutinin (PHA; Sigma) were used as positive controls at concentrations of 0·16 U/ml, 0·05 U/ml and 2 μg/ml, respectively. Cells were incubated for 6 days at 37°C in 5% CO_2, before being pulsed with 0·4 μCi ^{3}H-thymidine per well (Amersham, Aylesbury, UK) for 16 h. Cell proliferation was determined by measuring the incorporated radioactivity using liquid scintillation spectrometry (Liquid Scintillation Counter PW 4700; Philips, Eindhoven, The Netherlands). Results were expressed as stimulation indices (SI): the mean radioactivity, expressed as ct/min, incorporated by cells in the presence of antigen or mitogen, divided by the mean radioactivity (ct/min) incorporated in the absence of antigen. SIs of more than two were considered positive. The mean ct/min for the negative control latex beads was 370·7 (95% confidence interval: 261·6–479·9).

Serology

Serology was performed as described previously [17]. Briefly, microtitre plates were coated with crude *H. ducreyi* cocktail (5 μg/well). After blocking with newborn calf serum (GIBCO-BRL), adsorbed sera were diluted 1 : 200 and incubated at 37°C with antigen. Peroxidase-conjugated anti-human IgG, IgA or IgM was applied. The substrate solution consisted of H_2O_2 (Merck) and *o*-phenylenediamine (Sigma) in phosphate–citrate buffer pH 5·0. Colour development was allowed for 10 min at room temperature and OD was read at 490 nm. The cut-off value was defined as twice the negative control value. OD ratios < 0·85 (i.e. the respective OD of each sample divided by the cut-off value) were considered as negative, ratios more than 1·15 as positive, and ratios in the 0·85–1·15 range as undeterminate.

Statistical analysis

Because most data did not fit into a Gaussian distribution, the non-parametric Kruskal–Wallis test was systematically used to compare the results in the various subject groups.

RESULTS

Cellular immune responses in HIV⁻ chancroid patients

The PBMC from seven HIV⁻ chancroid patients, four successfully treated chancroid patients, two syphilis patients, and 15 healthy individuals with no history of chancroid, were stimulated with *H. ducreyi* antigen-coated beads, soluble TT or VZ antigen and PHA. All individuals responded well to PHA stimulation (SI median, 58·5; SI range, 3·1–437·4). Overall, the response to TT was poor (SI median, 0·9; SI range, 0·7–84·7) with 67% of the individuals tested responding with a stimulation index less than 2. In contrast, VZ antigen induced a clear cut memory type response (SI median, 14·0; SI range, 3·5–102·1) and was used as positive control antigen in further experiments.

Figure 1 shows stimulation profiles representative of untreated HIV⁻ patients with recent *H. ducreyi* infection

Table 3. Additional sample information for HIV⁻ individuals

No.	*Hd* cult.	*Hd* EIA	Syphilis (RPR)	T cell response to TT‡	VZ‡	PHA‡
Chancroid patients						
1	+	+*	−	0·85	ND	107·45
2	+	+	+	21·18	ND	78·9
3	+	+	−	1·39	ND	83·2
4	+	+	−	0·82	ND	21·9
5	+	−†	−	ND	60·04	58·5
Control individuals						
6	−	−	+	15·56	ND	120·9
7	−	−	+	1·05	ND	437·4
8	NA	−	NA	ND	11·85	43·09
9	NA	−	NA	ND	13·08	8·5
10	NA	−	NA	ND	42·39	97·2

Specimen numbers correspond to the numbers in Fig. 1.

NA, Not applicable; ND, not done; TT, tetanus toxin, VZ, Varicella/Zoster virus antigen; PHA, phytohaemagglutinin; EIA, enzyme immunoassay; RPR, Rapid Plasma Reagin test.

* Optical density ratio >1·15 in IgG, IgA or IgM EIA.

† Optical density ratio <0·85 in IgG, IgA and IgM EIA.

‡ Stimulation index.

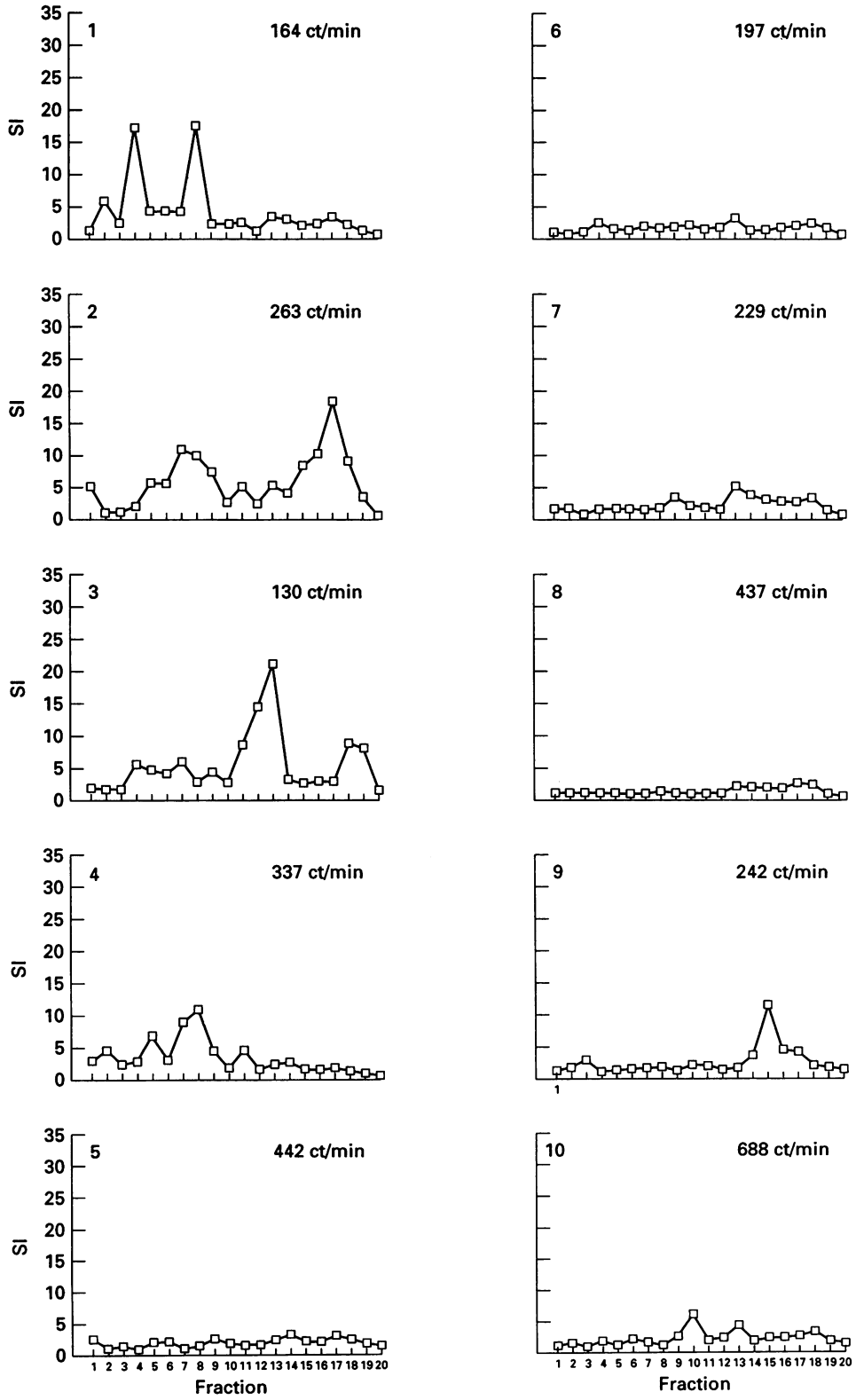

Fig. 1. Stimulation profiles from HIV$^-$ chancroid patients and controls. PBMC isolated from untreated (stimulation profiles 1–4) and treated (stimulation profile 5) HIV$^-$ chancroid patients, from syphilis control patients (stimulation profiles 6 and 7) and healthy control individuals (stimulation profiles 8–10) were stimulated with 20 different *Haemophilus ducreyi* antigen fractions coated on latex beads. Incubation was allowed for 6 days. Proliferation was measured by ^{3}H-thymidine uptake and results were expressed as stimulation index (SI). High molecular weight fractions starting from left. The ct/min value for the negative control latex beads is indicated for each individual in the right upper corner.

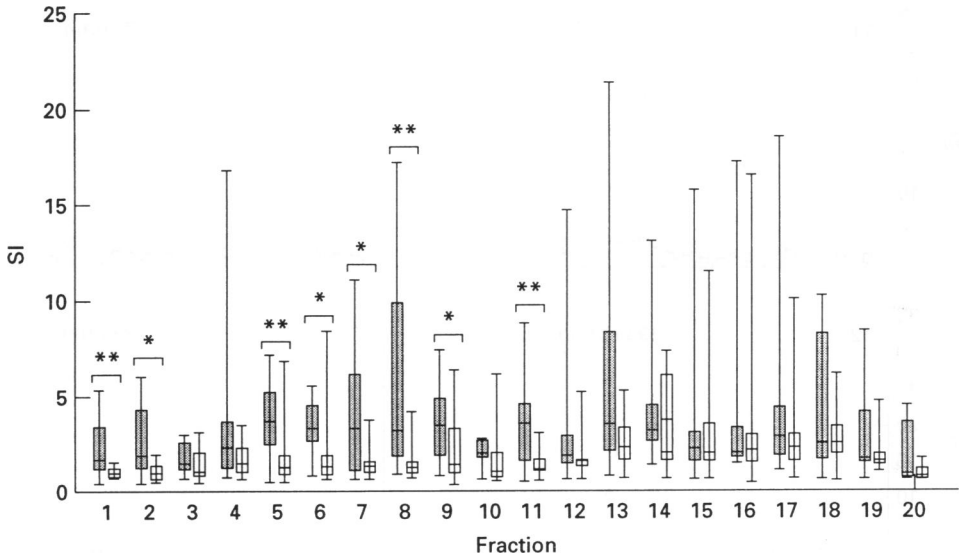

Fig. 2. Evaluation of the cellular immune response to *Haemophilus ducreyi* antigen-coated latex beads. PBMC from 11 HIV$^-$ chancroid patients (■) and 17 healthy control individuals (□) were stimulated with 20 different *H. ducreyi* antigen fractions coated on latex beads. Stimulation indexes (SI) were calculated and statistical analysis was performed. The boxes represent the 25th and 75th percentiles. The median and ranges are shown. Significant differences between stimulation in chancroid patients and controls are indicated (*$P < 0.05$; **$P < 0.01$).

(stimulation profiles 1–4), of successfully treated chancroid patients (stimulation profile 5), of patients with syphilis (stimulation profiles 6 and 7), and of healthy controls (stimulation profiles 8–10). In Table 3 additional information on these subjects is shown.

Compared with control individuals (stimulation profiles 6–10), PBMC from six out of seven untreated chancroid patients showed an increased proliferation to several antigen fractions (SI > 5), but significant variability was observed among individual chancroid patients. Only one patient did not show any stimulatory response. None of the individuals who had been successfully treated for chancroid a few months before blood was collected showed a significant proliferative response.

There was no association between values found in the different EIAs and the presence or absence of a certain proliferation peak.

Statistical analysis of these data revealed significant differences ($P < 0.05$) between the median SI of all HIV$^-$

Table 4. Additional sample information for HIV$^+$ individuals

No.	*Hd* cult.	*Hd* EIA	Syphilis (RPR)	T cell response to TT‡	VZ‡	PHA‡	CD4$^-$ counts
Chancroid patients							
11	–	+*	–	ND	2·7	49·4	188·2
12	–	+	+	ND	5·56	51·7	ND
13	–	+	–	ND	15·1	88·9	330·7
14	+	+	–	1·79	ND	149	384
15	+	+	–	ND	4·26	16·6	ND
Control individuals							
16	NA	–†	NA	ND	0·19	40·4	280
17	NA	–	NA	ND	2·30	77·5	780
18	NA	–	NA	ND	1·40	99·4	20
19	NA	–	NA	ND	1·33	7·0	290
20	NA	–	NA	ND	0·75	51·9	20

Specimen numbers correspond to the numbers in Fig. 3.

NA, Not applicable; ND, not done; TT, tetanus toxin; VZ, Varicella/Zoster virus antigen; PHA, phytohaemagglutinin; EIA, enzyme immunoassay; RPR, Rapid Plasma Reagin test.

* Optical density ratio >1·15 in IgG, IgA or IgM EIA.

† Optical density ratio <0·85 in IgG, IgA and IgM EIA.

‡ Stimulation index.

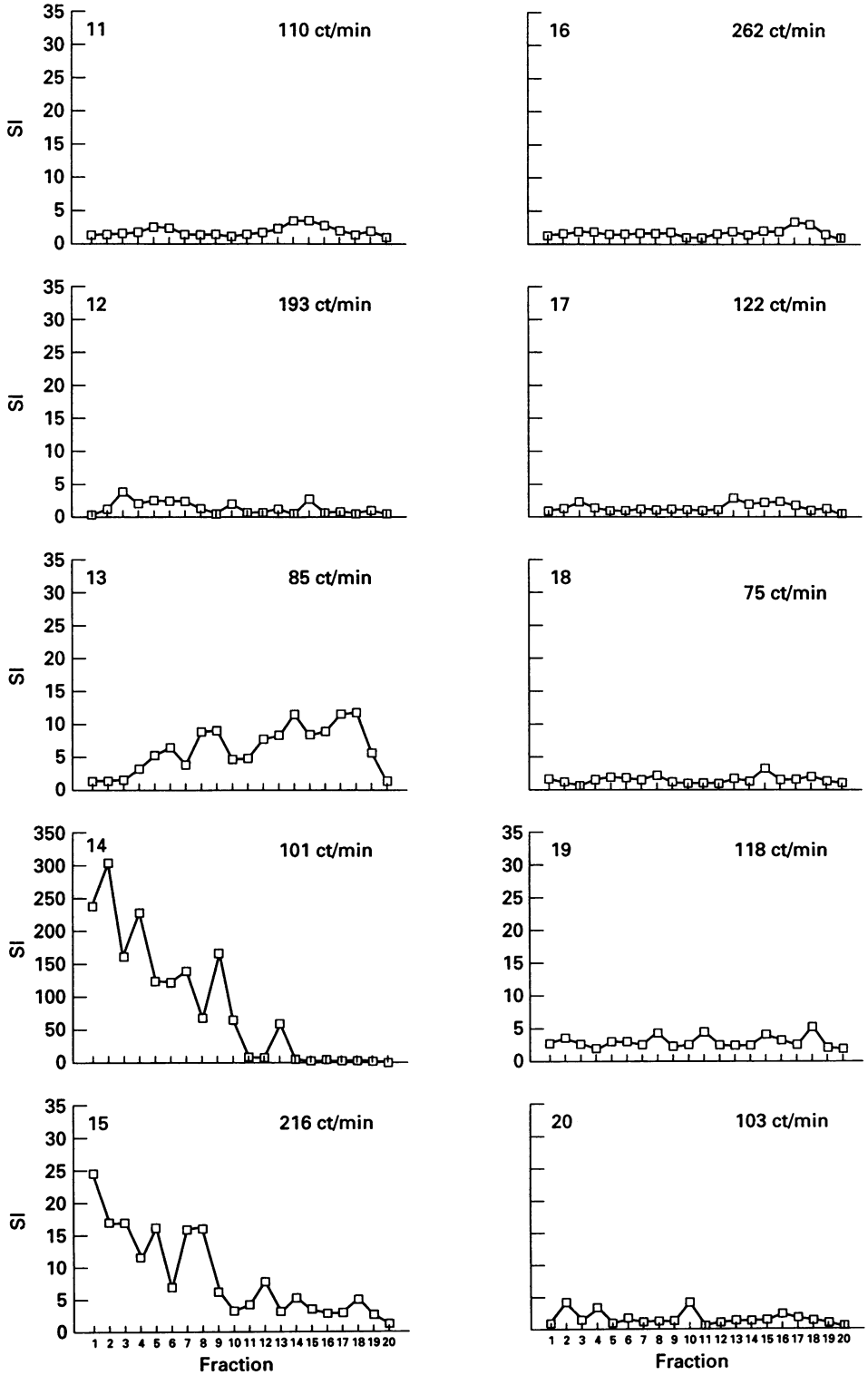

Fig. 3. Stimulation profiles of HIV$^+$ chancroid patients and controls. PBMC isolated from HIV$^+$ chancroid patients (stimulation profiles 11–15) and HIV$^+$ control individuals (stimulation profiles 16–20) were stimulated by 20 different *Haemophilus ducreyi* antigen fractions coated on latex beads. Experimental conditions and data representation are as in Fig. 1.

chancroid patients and the median SI of all HIV$^-$ control individuals, after stimulation with fractions 1 (> 91–91 kD), 2 (91–78 kD), 5 (59–51 kD), 6 (51–44 kD), 7 (44–38 kD), 8 (38–33 kD), 9 (33–29 kD) and 11 (25–21 kD) (Fig. 2).

Cellular immune responses in HIV$^+$ chancroid patients

With the exception of patient 14 who responded well to positive control antigens, HIV$^+$ patients responded weakly to VZ antigen stimulation (SI median, 2·5; SI range, 0·2–15·1)

compared with HIV^- individuals. Neither population differed significantly in terms of PHA stimulation (SI median, 49·4; SI range, 7·0–149·0). In eight out of 12 HIV^+ patients, CD4 counts were below the critical treshold of 400 (Table 4).

Figure 3 shows profiles representative of HIV^+ chancroid patients (stimulation profiles 11–15) and HIV^+ patients without *H. ducreyi* infection (stimulation profiles 16–20). Four out of seven tested HIV^+ chancroid patients showed a poor proliferative response, as shown in stimulation profiles 11 and 12. Three out of these four were *H. ducreyi* culture-negative, but strongly positive in *H. ducreyi* EIA. One HIV^+ chancroid patient (stimulation profile 13) reacted similarly to the HIV^- chancroid patients, showing clearly positive responses of limited magnitude ($5 < SI < 25$) distributed over several molecular weight fractions. But again, this patient was *H. ducreyi* culture-negative. Two *H. ducreyi* culture-positive patients (14 and 15) had an exceptional profile. The main stimulatory activity was restricted to the higher molecular size range. Patient 14 showed SIs > 200. The responses to *H. ducreyi* antigen-coated latex beads in non-chancroid HIV^+ patients (profile 16–20) were comparable to the responses observed in HIV^- controls (profile 6–10).

DISCUSSION

To our knowledge this is the first study providing *in vitro* evidence of the occurrence of a specific cellular immune response to *H. ducreyi* antigens in infected patients.

As observed in other infections, stimulation profiles differed greatly from one individual to another. Similarly, inter-patient differences were observed earlier for the humoral immune response to *H. ducreyi* infection [25,26]. These differences in immune response may be explained by antigenic diversity among *H. ducreyi* strains [19] and genetic differences between individuals. In addition, the site, frequency and duration of infection and the size of the inoculum may determine the proliferation profile.

Overall, control individuals were only weakly reactive to *H. ducreyi* antigen, irrespective of their ethnicity. The limited stimulatory activity was obtained with low molecular size components only. The four patients who were successfully treated for chancroid a few months before blood was collected did not show significant proliferative responses to *H. ducreyi* antigen. The apparent absence of a specific immune memory in these patients may explain the occurrence of recurrent infection. However, no blood was collected during actual *H. ducreyi* infection. Therefore, it remains possible that these patients did not show any lymphoproliferative response even during their active disease. In fact, a lack of stimulatory response was observed in one of the patients with acute disease as well. Alternatively, the laboratory isolates used to compose the crude antigen cocktail may no longer express important immunogenic proteins which were recognized by the immune system of these patients.

Statistical analysis of the PBMC responses from chancroid patients and controls to *H. ducreyi* antigen revealed several antigenic fractions with specific stimulatory activity. These fractions cover molecular sizes ranging from (i) 91–78 kD; (ii) 59–29 kD; and (iii) 25–21 kD. The lower molecular weight range apparently did not contain *H. ducreyi*-specific antigen, because comparable cell proliferation was induced in that area for chancroid patients as well as for control individuals. This response may be explained by non-specific polyclonal stimulation due to the presence of LPS-like substances.

The PBMC isolated from HIV^+ individuals reacted normally after mitogen stimulation, but had a decreased response to control antigen stimulation, as described by others [27]. Responses of PBMC from HIV^+ chancroid patients to *H. ducreyi* antigen were divergent. The low responses observed in four patients may be due to antigen-specific T cell dysfunction as a consequence of HIV infection [27,28]. One patient showed a profile similar to those found in HIV^- chancroid patients. Since the response of this patient to VZ was also normal, it seems that his immune system was still functional, in spite of the low CD4 counts. Two HIV^+ chancroid patients revealed extremely high SIs. The responses to PHA were not extreme compared with values found for HIV^- individuals. The *H. ducreyi*-induced proliferation was seen preferentially in the high molecular weight range, suggesting a specific response. It seems unlikely that a spontaneous and non-specific T lymphocyte proliferation [27] could explain this observation. The apparent specificity of the PBMC response also excludes an effect of an HIV-encoded superantigen [29]. However, *H. ducreyi*-specific proliferation may be increased in the same way as described by Pugliese *et al.* [30]. They discovered a peptide derived from HIV gp120 and functional homologous to the HLA-DR molecule, which was able to increase purified protein derivative-specific and autoreactive T cell proliferation.

Four out of five *H. ducreyi* culture-negative, *H. ducreyi* EIA-positive chancroid patients belonged to the HIV^+ patient group. One can expect some degree of non-specific stimulation of antibody production (hypergammaglobulinaemia) in HIV^+ patients. However, the specificity of the IgA-EIA (97%; 95% confidence interval: 95–99%) was reported to remain equally high in HIV^+ individuals [17]. This supported the decision to consider these cases as chancroid patients. Moreover, if the *H. ducreyi* culture-negative patients had been excluded from this study, it would appear as if most HIV^+ chancroid patients showed the exceptional profile discussed above.

As yet no data are available on lymphoproliferative responses in the temperature-dependent rabbit model [3], nor in the primate model [4] for chancroid. This study provides the basic information necessary to prove the validity of observations done in the suggested animal models for evaluation of the human system.

A number of limitations of this study are evident. No attempt was made to standardize the amount of protein bound to the latex beads. This may account partly for the dominant nature of protein fractions 8 and 5. Furthermore, each fraction still contained a mixture of different antigens. Thus, the individual contributions of the different proteins could not be determined. It may be that a protein of minor importance in terms of presence, can be a major immunogen. The use of purified proteins will help to overcome these limitations.

In conclusion, this study provides evidence for a *H. ducreyi*-induced cellular immune response in chancroid patients. In general, this response was apparent in HIV^- but not in HIV^+ chancroid patients. The development of an immune memory was not evident. Immunogenic determinants that may specifically stimulate cell proliferation were localized in terms of molecular size, but need to be identified.

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