Immunology 1989 **68** 325–331

Human monoclonal antibodies against a recombinant HIV envelope antigen produced by primary *in vitro* immunization. Characterization and epitope mapping

M. OHLIN,* P.-A. BROLIDEN,†‡ L. DANIELSSON,* B. WAHREN,† J. ROSEN,§ M. JONDAL‡ & C. A. K. BORREBAECK* *Department of Biotechnology, Lund University, Lund, †Department of Virology, National Bacteriological Laboratory, Stockholm, ‡Department of Immunology, Karolinska Institute, Stockholm, Sweden and §Johnson & Johnson Biotechnology Center, La Jolla, California, U.S.A.

Accepted for publication 7 July 1989

SUMMARY

Peripheral blood lymphocytes from healthy, HIV sero-negative blood donors have been *in vitro* immunized using penv9, a recombinant fragment of the envelope of HIV-1. This primary *in vitro* immunization followed by Epstein–Barr virus (EBV) transformation and somatic cell fusion subsequently gave rise to several specific anti-penv9 monoclonal antibodies (MO28, MO30 and MO43) of μ isotype. The hybridomas have been kept in culture for over 6 months and the antibody productivity for MO30 was measured to 18 $\mu g \times (24\ hr \times 10^6\ cells)^{-1}$. The fine specificity of the antibodies was mapped by a peptide inhibition enzyme immunoassay, using overlapping synthetic pentadeca peptides covering the whole penv9. These human monoclonal antibodies exhibited a similar epitope specificity directed against a non-sequential determinant, including the amino acids 632–646, 677–681 and 687–691. This specificity is very rarely found in immune sera from sero-positive patients and presently not reported in human monoclonal antibodies derived from *in vivo* immunized individuals, indicating that different antibody specificities can be obtained by the *in vitro* immunization technology. These human monoclonal antibodies did not neutralize HIV. The results presented here demonstrate the feasability of generating human monoclonal antibodies against HIV by primary *in vitro* immunizations, thereby avoiding the use of lymphocytes derived from infected patients when human monoclonal antibodies for therapeutic purposes are to be produced.

INTRODUCTION

Human immunodeficiency virus (HIV) is the etiological agent for the acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi *et al.*, 1983; Coffin *et al.*, 1986; Gallo *et al.*, 1984; Wong-Staal & Gallo, 1985). The humoral immune response to HIV has been investigated extensively (Ho *et al.*, 1987; Sarngadharan *et al.*, 1984) and serum antibodies capable of inhibiting viral infection and syncytia have been identified in serum of HIV-infected individuals (Lifson *et al.*, 1986; Weiss *et al.*, 1985, 1986). The major epitopes involved in neutralizing HIV-1 appear in gp120 of the virus envelope (Ho *et al.*, 1987; Palker *et al.*, 1988; Putney *et al.*, 1986; Rusche *et al.*, 1988), whereas minor neutralization-inducing epitopes are present in the transmembraneous glycoprotein gp41 (Ho *et al.*, 1987). Neutralizing antibodies have also been described after immunization with peptides representing stretches of gp41 (Dalgleish *et al.*, 1988; Schrier *et al.*, 1988). Furthermore, decrease in neutralizing antibody titres and reactivity against HIV gag-antigen have been shown to correlate with the clinical progression of infected patients (Weber *et al.*, 1987), although no definitive correlation has been found between the presence of neutralizing antibodies and clinical disease.

Neutralizing antibodies against viral antigens have shown protective effects in patients infected with cytomegalovirus (Blacklock *et al.*, 1985) and in prevention of viral infection by prophylaxis (Condie & O'Reilly, 1984) and might have a similar beneficial effect on the clinical course of HIV infections. Recent data on antibody-mediated therapy using human polyclonal antibodies against HIV envelope proteins has, however, not shown the desired degree of protection, when tested prophylactically in chimpanzees subsequently challenged with HIV (Prince *et al.*, 1988). To ultimately evaluate antibody-mediated therapy, the use of human monoclonal antibodies directed against envelope antigens has to be examined in HIV-infected patients. It has also been suggested that human monoclonal antibodies recognize epitopes that the mouse immune system neglects (Borrebaeck, 1988; Effros *et al.*, 1986), which might prove advantageous in a therapeutic situation. However, due to present regulations of immunotherapy it is hard to envisage a

Correspondence: Dr C. A. K. Borrebaeck, Dept. of Biotechnology, Lund University, P.O. Box 124, S-22100 Lund, Sweden.

clinical *in vivo* use of human monoclonal antibodies produced from lymphocytes of HIV-infected patients. This problem might be circumvented by *in vitro* immunization (Borrebaeck, 1988; Borrebaeck, Danielsson & Möller, 1988). We report here on the production of human monoclonal antibodies against penv9, a recombinant protein of gp160 of HIV-1, using healthy blood donor lymphocytes immunized *in vitro* and the subsequent epitope mapping of these antibodies.

MATERIALS AND METHODS

Antigen, mitogens and peptides

Recombinant IL-2 was obtained from Sandoz Research Institute (Vienna, Austria). The recombinant proteins env9 (Putney *et al.*, 1986) and env17 produced in *Escherichia coli* were generous gifts from Drs S. Erickson-Viitanen and S. R. Petteway. (E.I. DuPont de Nemours & Company, Wilmington, DE). Pokeweed mitogen (PWM) and supernatant from the Epstein–Barr virus (EBV)-producing marmoset cell line B95-8 were kindly supplied by Dr J. Börjesson (Clinical Chemistry, Helsingborg Hospital, Sweden) and by Dr A. Rosén (Karolinska Institute, Stockholm, Sweden), respectively. Overlapping synthetic pentadeca-peptides covering the sequence of env9 protein were obtained from Johnson & Johnson Biotechnology Center, La Jolla, CA.

Cells

Buffy coats from registered sero-negative blood donors were obtained from Lund University Hospital Blood Bank (Lund, Sweden). Peripheral blood lymphocytes (PBL) were prepared by density centrifugation on Ficoll–Paque (Pharmacia Fine Chemicals AB, Uppsala, Sweden) and washed twice in phosphate-buffered saline.

Treatment of PBL with L-leucyl-L-leucine methyl ester

Treatment of peripheral blood lymphocytes with L-leucyl-L-leucine methyl ester hydrobromide (LeuLeu-OMe) (Bachem Feinchemikalien AG, Budendorf, Switzerland) was performed using a modification of a previously described method (Borrebaeck, Danielsson & Möller, 1987; Borrebaeck *et al.*, 1988). Briefly, washed lymphocytes isolated by density centrifugation were incubated with freshly prepared 0·25 mM LeuLeu-OMe in RPMI-1640 for 15 min at room temperature. The cells were then washed three times with culture medium containing 2% heat-inactivated (30 min, 56°) human serum (Lund University Hospital Blood Bank).

In vitro *immunization*

The *in vitro* immunization was performed as described previously (Borrebaeck *et al.*, 1988), using LeuLeu-OMe-treated PBL in RPMI-1640 containing 10% human serum, 4 mM L-glutamine, 1% (v/v) 100 × non-essential amino acids and 50 μg gentamicin/ml (Flow Laboratories Inc., Irvine, Ayrshire, U.K.). During *in vitro* immunization, the medium was further supplemented with 50 μM 2-mercaptoethanol, 5 IU recombinant IL-2, antigen (25–500 ng/ml) and 25% (v/v) supernatant, isolated from a culture of irradiated (2000 rads) human T cells stimulated over night with 10 μg PWM/ml (Danielsson, Möller & Borrebaeck, 1987). The immunization period was 6 days at 37° in a humidified incubator with 8% CO_2 and 92% air gas phase.

Infection of lymphocytes with EBV

The EBV-containing supernatant was filtered through a 0·45 μm sterile filter, stored at 4° and was used without further treatment. Prior to infection with EBV, *in vitro* immunized lymphocytes were washed twice with serum-free RPMI-1640. Cells were infected for 2 hr at 37° with occasional stirring using 1 ml of EBV-containing supernatant per 10^7 lymphocytes. After infection the cells were washed twice with RPMI-1640 medium, containing 10% fetal calf serum (FCS). Finally, infected cells were seeded at 10^5 cells/well in 96-well microtitre plates with feeder cells [10^4 irradiated (3000 rads) PBL per well] in supplemented RPMI-1640, containing 10% FCS (Gibco Ltd, Paisley, Renfrewshire, U.K.).

Establishing antigen-specific EBV-transformed lymphoblastoid cell lines (LCL)

Primary LCL were tested for antigen-specific reactivity using an enzyme-linked immunosorbent assay (ELISA) after 3–5 weeks, and positive LCL were expanded to 24-well plates and retested for specific antibody production. Selected LCL were further expanded for subsequent somatic cell fusion or were cloned at 10–1000 cells/well with feeder cells [10^4 irradiated (3000 rads) PBL per well].

Fusion and cloning of cells

In vitro immunized PBL and the human × mouse heterohybridoma K6H6/B5 (Caroll *et al.*, 1986), at a ratio of 2:1, were fused in medium containing 45% (v/v) polythylene glycol (1540, MW PEG-1540) and 7·5% dimethyl sulphoxide, as described previously (Borrebaeck *et al.*, 1988). Cells were seeded at 3×10^5 cells/well in 96-well plates in supplemented RPMI-1640, containing 10% FCS, 100 μM hypoxanthine, 0·4 μM aminopterin and 16 μM thymidine (HAT medium). Clones were tested with ELISA for antigen-specific antibodies after 2–4 weeks.

Expanded lymphoblastoid cell lines were also fused as soon as possible with the heteromyeloma K6H6/B5, at a ratio of 1:1. The fusion was performed as described above and cells were seeded at $1–3 \times 10^5$ cells/well in 96-well plates in HAT medium, containing 1 μM ouabain (Sigma Chemical Co., St Louis, MO). Clones were tested with ELISA for antigen-specific antibodies after 2–4 weeks. Antigen-positive wells were expanded to 24-well plates and retested after a few days for sustained antibody production. Cloning by limiting dilution was performed at least twice, using 10^4 irradiated (3000 rads) PBL per well as feedercells.

Partial purification of human monoclonal IgM

Cell culture supernatants were concentrated ten times by ultrafiltration (PM10, Amicon Co., Danvers, MA) and proteins were precipitated twice with 16% PEG 6000, as described elsewhere (Neoh *et al.*, 1986). The precipitate was dissolved in 10 mM phosphate buffer, pH 7·2, containing 0·3 M NaCl.

Analytical procedures

Antigen-specific ELISA for screening purposes was performed by coating antigen (100 ng penv9/well) in a 50 mM sodium carbonate buffer, pH 9·6, in 96-well ELISA microtitre plates (M129B) (Dynatec Labs Ltd, Billingshurst, Sussex, U.K.). Hybridoma supernatants and horseradish peroxidase conjugated to rabbit anti-human immunoglobulins (Dakopatts A/S, Glostrup, Denmark) were diluted in 10 mM sodium phosphate, pH 8·0, containing 0·5 M sodium chloride and 0·1% Tween 20.

Table 1. Immortalization frequencies and number of antigen-specific cell lines after *in vitro* immunization and subsequent EBV-transformation or somatic cell fusion using K6H6/B5

Exp. no.	Antigen conc. (ng/ml)	Immortalization	Cell growth*	Specific growth†	Antigen-specific cell lines/10^6 lymphocytes‡
1§	0	EBV¶	192/192	1/192	0·05
	0	Fusion**	133/192	0/133	<0·02††
	25	EBV	192/192	8/192	0·4
	25	Fusion	125/192	0/125	<0·02
	25	Fusion	446/480	0/446	<0·01
	250	EBV	192/192	4/192	0·2
	250	Fusion	ND	ND	0·03
2	500	Fusion	538/576	1/538	0·008
3	50	Fusion	102/123	1/102	0·04
	500	Fusion	136/237	3/136	0·06

* Number of growth-positive wells/number wells seeded.

† Number of antibody-positive wells/number growth-positive wells. The antibody-positive cell lines had an $A_{405} \geq 0{\cdot}8$.

‡ Calculated by Poisson distribution.

§ Immortalizations in each experiment were performed using the same lymphocyte batch.

¶ EBV-transformed *in vitro* immunized lymphocytes were seeded at 10^5 cells/well and grown with irradiated (3000 rads) PBL as feeder cells.

** Fused cells were seeded at 2×10^5 lymphocytes/well.

†† No antigen-specific clones were obtained.

ND, not determined.

ABTS [2,2′-azino-di(3-ethyl benzthiazoline sulphonic acid) (0·16 mM)] or o-phenylene diamine (0·7 mg/ml) together with hydrogen peroxide (2 mM) was used as enzyme substrate. Antibody specificity was tested, with the same screening ELISA, against control antigens such as: penv17 (200 ng/well), human serum albumin (0·1 or 1 μg/well), DNP-conjugated to human serum albumin (ratio 5:1) (0·1 or 1 μg protein/well), bovine serum albumin (1 μg/well), gelatin (1 μg/well), human transferrin (0·1 or 1 μg/well), or keyhole limpet haemocyanin (1 μg/well).

Quantification of immunoglobulin isotypes was performed by a standard ELISA procedure, using isotype-specific affinity-purified antibodies obtained from Zymed Laboratories Inc. (San Francisco, CA). Briefly, 50 ng/well of affinity-purified, isotype-specific antibodies, dissolved in 50 mM sodium carbonate buffer, pH 9·5, were coated in 96-well microtitre plates. Samples, Ig-standard (Dakopatts A/S) and horseradish peroxidase conjugated to isotype-specific antibodies (40 ng/well) were all diluted in 10 mM sodium phosphate buffer, pH 8·0, containing 0·15 M sodium chloride and 0·1% Tween 20. O-phenylenediamine was used as enzyme chromogen. All washing steps were performed using 0·15 M sodium chloride, containing 0·05% Tween 20 (Borrebaeck, 1983).

Determination of antibody-dependent cellular cytotoxicity (Ljunggren *et al.*, 1987), complement-dependent cytotoxicity and virus neutralization (Wahren *et al.*, 1988) were performed as previously described.

The antibody binding site on penv9 was mapped by inhibition ELISA (Niedrig *et al.*, 1989) using overlapping pentadecapeptides (2·5 μg/ml), synthesized from sequences of HTLV IIIB, clone 10 (Ratner *et al.*, 1985) and covering the entire penv9 sequence. Each peptide overlapped the previous peptide by 10 amino acids. The amino acid numbers follow the Los Alamos Database (Myers *et al.*, 1988).

Determination of DNA sequences

RNA isolated from MO30 hybridoma cells was used for cDNA synthesis. The cDNA was used as template for primer-directed DNA synthesis (polymerase chain reaction), resulting in specific amplification of sequences encoding immunoglobulin variable regions. The amplified cDNA was cloned into M13 sequencing vectors, and the DNA sequence determined using the dideoxy chain-termination reaction. Details of the procedure and the variable region sequence were recently published by Larrick *et al.* (1989).

Analysis of hydrophilicity

Hydrophilicity plots were constructed for the amino acid sequence deduced from DNA sequences of the MO30 μ chain variable region and of the penv9 region of the HIV genome (Ratner *et al.*, 1985), respectively. The calculations were performed according to Hopp & Woods (1981) on hexapeptide amino acid sequences.

RESULTS

EBV-transformation or cell fusion of *in vitro* immunized PBL

The *in vitro* immunizations were performed for 6 days (Borrebaech *et al.*, 1988) using 0, 25, 50, 250 and 500 ng penv9/ml. The results of *in vitro* immunization have been evaluated by the number of antigen-specific cell lines obtained after EBV transformation and/or somatic cell fusion, as determined by the screening ELISA. The frequency of antigen-specific cell lines was dependent on the antigenic dose present during the *in vitro* immunization period and most cell lines were obtained when 25 ng/ml was used during *in vitro* immunization and when EBV was used in the immortalization step (Table 1). It was evident that

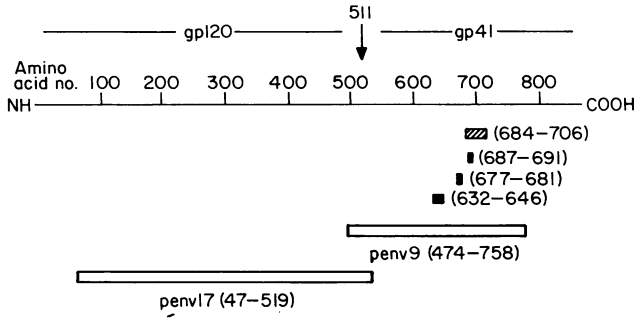

Figure 1. Schematic presentation of the envelope proteins and the recombinant peptides of the human immunodeficiency virus. The transmembrane region of gp41 (▨) and the amino acid sequences of the antibody epitopes (■) are shown. Arrow indicates the amino terminal end of gp41.

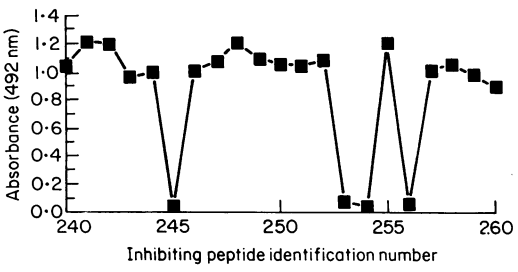

Figure 2. An epitope-mapping of MO43 by inhibition ELISA using overlapping synthetic pentadecapeptides covering the entire penv9 sequence. Only the region where inhibition was obtained is shown. The peptides are numbered for identification and the corresponding amino acid sequence of each epitope is given in the text.

the procedure for immortalization of antigen-specifically activated B cells was critical for the probability of securing continuous monoclonal antibody production. The frequency of antigen-specific cell lines was 10–20 times higher when using EBV-transformation of *in vitro* immunized lymphocytes compared with polyethylene-induced somatic cell fusion. In comparison, the frequency of antigen-specific cell lines derived from EBV transformation of *in vitro* immunized human splenocytes (100 ng penv9/ml) was $<0{\cdot}05 \times 10^{-6}$. All human monoclonal antibodies obtained from PBL directed against penv9 were of the μ isotype with κ light chains.

Expansion, fusion and cloning of EBV-transformed lymphoblastoid cells

In vitro immunized and EBV-transformed lymphocytes were tested after 3–5 weeks using the screening ELISA, specific for penv9. Cells from tissue culture wells containing antigen-specific LCL (92/1440) were expanded to 24-well culture plates and retested after 3–5 days for sustained antibody production by the ELISA. Of the replated LCL, 53% (49/92) remained strongly ELISA positive after the expansion and 2% (2/92) showed a non-specific pattern of reaction. Nineteen replated LCL with high antibody titres were selected and reactivity was tested against the recombinant env17 (amino acids 47–519) (Ratner *et al.*, 1985), covering most of gp120 and a minor N-terminal part of gp41 of HIV-1 (Fig. 1). None of these LCL showed any reactivity with penv17 and six of them (6/19) were cloned or further expanded and fused with the heteromyeloma K6H6/B5.

Expanded, antigen-specific master well cultures or cloned EBV-transformed cell lines ($2–20 \times 10^6$ cells) were fused to K6H6/B5 using polyethylene glycol. The frequency of resulting viable hybrids varied between 10^{-4} and 10^{-6} ($n=19$). It was noted that the obtained frequency of antigen-specific clones after fusion seemed to be related to the initial antibody titre in the supernatant of the EBV-transformed cell line, i.e. high ELISA titre resulted in high frequency of antigen-specific clones. Furthermore, the subsequent cloning efficiency of hybridomas was drastically improved by the addition of 10^4 irradiated feeder cells (PBL) per well. No additional improvement in cloning efficiency was obtained when the number of feeder cells was further increased 10 times. A cloning efficiency of $22 \pm 2{\cdot}5$ (SEM)% ($n=28$) was normally achieved when 1–3 cells were cloned per well, using PBL as feeder cells.

Production, partial purification and specificity test of human IgM monoclonal antibodies

Static cultures of the human hybridomas secreting specific anti-penv9 antibodies resulted in an immunoglobulin concentration in spent medium of 10–20 μg/ml. One human hybridoma (MO30) grown in culture for over 6 months had a productivity of 18 $\mu g \times (24 \text{ hr} \times 10^6 \text{ cells})^{-1}$. After concentration by ultrafiltration the antibodies were quantitatively (>95% of antibody reactivity) recovered after two 16% polyethylene glycol precipitations.

The specificity of purified and reconstituted human monoclonal antibodies (MO28/MO30/MO43, diluted 1:1000) was determined by testing these antibodies in the ELISA against a number of unrelated proteins, such as penv17, human serum albumin, DNP conjugated to human serum albumin (5:1 ratio), bovine serum albumin, gelatin, human transferrin and keyhole limpet haemocyanin (KLH). No reactivity, above background values (OD 0·1–0·2), was obtained against any of the unrelated antigens, whereas when antibodies were tested against penv9 ELISA absorbance values of >4·5 were consistently achieved.

Epitope mapping of human monoclonal antibodies

The ELISA reactivity of antibodies MO28, MO30 and MO43 against penv9 was challenged by synthetic pentadecapeptides covering amino acid region 511–856 of gp41, using an inhibition ELISA. Figure 2 shows the inhibitory effect of peptides nos 245 and 253, 254 and 256 on the reactivity of MO43 monoclonal antibody against penv9. Peptide 245 corresponds to amino acid no. 632–646 and peptides 253, 254 and 256 correspond to amino acid nos 672–686, 677–691 and 687–701, respectively (Myers *et al.*, 1988).

These peptides identified an epitope having an amino acid sequence most likely in the range 632–646 (peptide no. 245), 677–681 (peptides nos 253–254) and 687–691 (peptides nos 254 and 256), located close and into the transmembrane region of gp41 (amino acids 684–706) (Fig. 1). All three human monoclonal antibodies, MO28, MO30 and MO43, showed a similar inhibition pattern, although they originally were derived from different master wells. No other peptides showed any inhibition

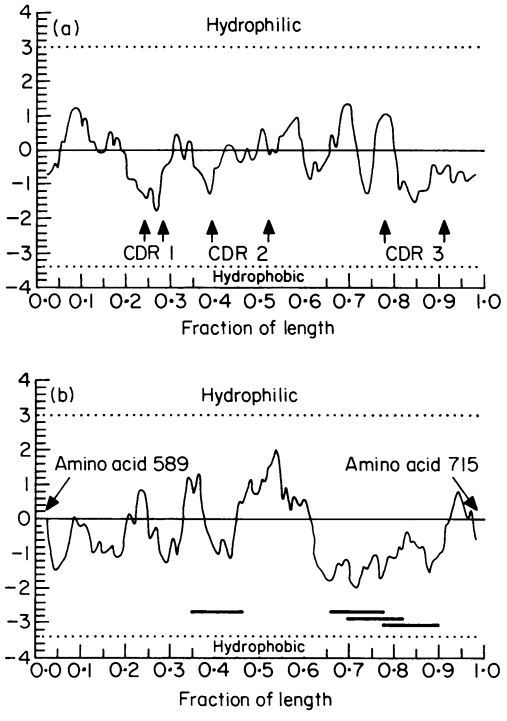

Figure 3. Hydrophilicity plots of the μ-chain variable region of antibody MO30 (a) and of the part of penv9 containing the non-sequential epitope (b). The location of complementarity-determining regions (CDR 1–3) of the antibody heavy chain and of the antibody-inhibiting peptides of penv9 are indicated.

of the monoclonal antibody reactivities, indicating that the specificity of MO28, MO30 and MO43 was most probably directed against a non-sequential epitope consisting of three hydrophobic regions (Muesing *et al.*, 1985); the same peptides did not inhibit other irrelevant human monoclonal anti-virus antibodies produced by *in vitro* immunizations. To further characterize antibody and antigen, we performed hydrophilicity plots of the region of penv9 that contained the antigenic epitopes and of the complementarity determining regions (CDR 1–3) of the heavy chain variable region of MO30. The hydrophobic nature of these regions was evident (Fig. 3); the light chain variable region showed similar hydrophobicity (data not shown). It should, however, be noted that a number of hydrophobic peptides from other regions of peny9 did not inhibit the antibodies, which supported that the inhibition shown in Fig. 2 was specific.

The antibodies MO28, MO30 and MO43 reacted with penv9 in dot blots (data not shown), although they did not react in Western blots with native gp41 using commercially available strips. The antibodies did not mediate antibody-dependent cellular cytotoxicity or neutralization of viral infections. Mapping of the epitopes on penv9 revealed, however, a monoclonal antibody specificity that very rarely has been observed even in polyclonal immune sera from HIV-infected patients (B. Wahren, unpublished data). This supports the important fact that different antibody specificities might be obtained in human monoclonal antibodies using recombinant antigens for *in vitro* immunization compared to when *in vivo* sensitized B cells are employed for antibody production.

DISCUSSION

Primary *in vitro* immunization of human peripheral blood lymphocytes has been shown to be a successful approach for the production of human monoclonal antibodies against a viral envelope antigen associated with the immunodeficiency virus. If therapeutically valuable human antibodies shall be produced the use of lymphocytes derived from sero-converted patients (Banapour *et al.*, 1987) cannot be utilized due to governmental regulatory reasons. *In vitro* immunization will consequently be the desired way for production of human monoclonal antibodies against HIV antigens since *in vivo* sensitization of healthy donors using recombinant viral antigens seems like a very unlikely approach. Replacement of complementarity-determining regions of a human antibody with those from a mouse monoclonal antibody (Riechmann *et al.*, 1988), directed against HIV antigens, may be another approach to avoid lymphocytes from infected patients, although the specificity of the chimaeric antibody will always be determined by the murine immune system, which may neglect epitopes that the human immune system would have recognized (Borrebaeck, 1988; Effros *et al.*, 1986).

It is now known that most infected patients have a strong sero-reactivity to a conserved and immunodominant region of gp41 (Chang *et al.*, 1985; Gnann, Nelson & Oldstone, 1987a; Smith *et al.*, 1987; Wang *et al.*, 1986), which is hidden in the native molecule (Närvänen *et al.*, 1988). Although this region is included in penv9 no reactivity was found against this conserved region in our human monoclonal antibodies, as determined by the peptide inhibition ELISA. However, human monoclonal antibodies produced using *in vivo* immunized B cells isolated from sero-positive patients exhibited a specificity directed against the conserved and immunodominant region of gp41 (R. von Baehrs *et al.*, personal communication). The human monoclonal antibodies that we report here do all exhibit similar epitope specificity; despite the fact that the clones were obtained from different masterwells it cannot be excluded that they represent subclones of the same B cell, since they all originated from the same *in vitro* immunization. The antibody specificity has, however, to our knowledge not been found in monoclonal antibodies derived from sero-positive patients (Gnann *et al.*, 1987b; Modrow *et al.*, 1987; Wang *et al.*, 1986), suggesting that other antibody specificities can be obtained using *in vitro* immunization compared to *in vivo* sensitization. This fact might prove important when specificities of human monoclonal antibodies neutralizing a multitude of HIV-1 isolate are sought, since functionally associated epitopes might be less immunogenic *in vivo*. We are presently investigating the possibility of producing neutralizing human monoclonal antibodies against regions of gp120 using primary *in vitro* immunization.

The optimal production technology for human monoclonal antibodies was clearly the combination of *in vitro* immunization of PBL with EBV transformation and subsequent fusion with a heteromyeloma. The approach of using synthetic peptides in an inhibition ELISA made it possible to reveal an apparent discontinuous epitope of gp41. This had gone undetected by other assays since the human monoclonal antibodies did not react with the peptides directly coated onto an ELISA plate (J. Hinkula *et al.*, personal communication). Several of the cloned lymphoblastoid cell lines, continuously secreting human monoclonal antibodies, did not exhibit EBNA (Epstein–Barr nuclear

antigen) (data not shown). There was, furthermore, no apparent reason for using human spleen cells in the *in vitro* immunizations instead of the easily accessible human peripheral blood lymphocytes, since the frequency of antigen-specific clones obtained from splenocytes was not high compared with PBL. It is also worth noticing that the EBV indeed infected and transformed human B cells that had been *in vitro* activated for 6 days in culture. These results are in contradiction to early findings that EBV only infect small, resting B cells (Åman, Ehlin-Henriksson & Klein, 1984) but are supported by more recent rapports addressing this particular problem (Chan *et al.*, 1986; Ohlin *et al.*, 1989).

In summary, we have applied the *in vitro* immunization technology to peripheral blood lymphocytes, derived from healthy blood donors, for the production of human monoclonal antibodies against an envelope protein of HIV-1. The epitopes have been mapped and the *in vitro* immunization technology seems to have the ability to give raise to antibody specificities not easily found in sera from infected patients.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Cancer Society, Nordisk Industrifond, the National Swedish Board for Technical Development, Medical Faculty (Lund University), BioInvent International AB, Magnus Bergvall Foundation, John and Augusta Persson Foundation, Österlunds Foundation, Ellen, and Walter and Lennart Hesselmans Foundation (C.A.K. Borrebaeck) and by a grant from the Swedish Medical Research Council (B. Wahren). We thank Eva Birkedal and Ann-Charlott Nilsson for skilfully performing the extensive ELISA screening and Jorma Hinkula for expert advice on the epitope mapping assay.

REFERENCES

Åman P., Ehlin-Henriksson B. & Klein G. (1984) Epstein–Barr virus susceptibility of normal human B lymphocyte populations. *J. exp. Med.* **159,** 208.

Banapour B., Rosenthal K., Rabin L., Sharma V., Young L., Fernandez J., Engleman E., McGrath M., Reyes G. & Lifson J. (1987) Characterization and epitope mapping of a human monoclonal antibody reactive with the envelope glycoprotein of human immunodeficiency virus. *J. Immunol.* **139,** 4027.

Barre-Sinoussi F., Chermann J.C., Rey F., Nugeyre M.T., Chamaret S., Gruest J., Dauguet C., Axler-Blin C., Vezinet-Brun F., Rouzioux C., Rozenbaum W. & Montagnier L. (1983) Isolation of a T lymphotropic retrovirus from a patient at risk for aquired immunodeficiency syndrome (AIDS). *Science,* **220,** 868.

Blacklock H.A., Griffiths P., Stirk P. & Prentice H.G. (1985) Specific hyperimmune globulin for cytomegalovirus pneumonitis. *Lancet.* **ii,** 152.

Borrebaeck C.A.K. (1983) *In vitro* immunization for the production of antigen-specific lymphocyte hybridomas. *Scand. J. Immunol.* **18,** 9.

Borrebaeck C.A.K. (1988) Human monoclonal antibodies produced by *in vitro* immunization. *Immunol. Today,* **9,** 355.

Borrebaeck C.A.K., Danielsson L. & Möller S.A. (1987) Human monoclonal antibodies produced from L-leucine methyl ester-treated and *in vitro* immunized peripheral blood lymphocytes. *Biochem. Biophys. Res. Commun.* **148,** 941.

Borrebaeck C.A.K., Danielsson L. & Möller S.A. (1988) Human monoclonal antibodies produced by primary *in vitro* immunization of peripheral blood lymphocytes. *Proc. natl. Acad. Sci. U.S.A.* **85,** 3995.

Caroll W.L., Thielemans K., Dilley J. & Levy R. (1986) Mouse × human heterhybridomas as fusion partners with human B cell tumors. *J. immunol. Meth.* **89,** 61.

Chan M.A., Stein L.D., Dosch H.-M. & Sigal N.H. (1986) Heterogeneity of EBV-transformable human B lymphocyte populations. *J. Immunol.* **136,** 106.

Chang T.W., Kato I., McKinney S., Chanda P., Barone A.D., Wong-Staal F., Gallo R.C. & Chang N.T. (1985) Detection of antibodies to human T-cell lymphotropic virus-III (HTLV-III) with an immunoassay employing a recombinant Escherischia coli-derived viral antigenic peptide. *Biotechnol.* **3,** 905.

Coffin J., Haase A., Levy J.A., Montagnier L., Oroszlan S., Teich N., Temin H., Toyoshima K., Vamus H., Vogt P. & Weiss R. (1986) Human immunodeficiency viruses. *Science,* **232,** 697.

Condie R.M. & O'Reilly R.J. (1984) Prevention of cytomegalovirus infection by prophylaxis with an intravenous, hyperimmune, native, unmodified cytomegalovirus globulin. Randomized trial in bone marrow transplant recipients. *Am. J. Med.* **76,** 134.

Dalgleish A.G., Chanh T.C., Kennedy R.C., Kanda P., Clapham P.R. & Weiss R.A. (1988) Neutralization of diverse HIV-1 strains by monoclonal antibodies raised against a gp41 synthetic peptide. *Virology,* **165,** 209.

Danielsson L., Möller S.A. & Borrebaeck C.A.K. (1987) Effect of cytokines on specific *in vitro* immunization of human peripheral B lymphocytes against T-cell dependent antigens. *Immunology,* **61,** 51.

Effros R.B., Hulette C.M., Ettinger R., Dillard L.C., Zeller E., Duong R. & Walford R. (1986) A human-human hybridoma secreting anti-HLA class II antibody. *J. immunol. Meth.* **137,** 1599.

Gallo R.C., Salahuddin S.Z., Popovic M., Shearer G.M., Kaplan M., Haynes B.F. *et al.* (1984) Frequent detection and isolation of cytopathic retrovirus (HTLV-III) from patients with AIDS and at risk for AIDS. *Science,* **224,** 500.

Gnann J.W., Nelson J.A. & Oldstone M.B.A. (1987a) Fine mapping of an immunodominant domain in the transmembrane glycoprotein of human immunodeficiency virus. *J. Virol.* **61,** 2639.

Gnann J.W., Schwimmbeck P.L., Nelson J.A., Truax A.B. & Oldstone M.B.A. (1987b) Diagnosis of AIDS by using a 12-amino acid peptide representing an immunodominant epitope of the human immunodeficiency virus. *J. Infect. Dis.* **156,** 261.

Ho D.D., Sarngadharan M.G., Hirsch M.S., Schooley R.T., Rota T.R., Kennedy R.C., Chanh T.C. & Sato V.L. (1987) Human immunodeficiency virus neutralizing antibodies recognize several conserved domains on the envelope glycoproteins. *J. Virol.* **61,** 2024.

Hopp T.P. & Woods K.R. (1981) Prediction of protein antigenic determinants from amino acid sequences. *Proc. natl. Acad. Sci. U.S.A.* **78,** 3824.

Larrick J.W., Danielsson L., Brenner C., Abrahamson M., Fry K. & Borrebaeck C.A.K. (1989) Rapid cloning of rearranged immunoglobulin genes from human hybridoma cells using mixed primers and the polymerase chain reaction. *Biochem. Biophys. Res. Commun.* **160,** 1250.

Lifson J.D., Feinberg M.B., Reyes G.R., Rabin L., Banapour B., Chakrabarti S., Moss B., Wong-Staal F., Steimer K.S. & Engleman E.G. (1986) Induction of CD4-dependent cell fusion by the HTLV-III/LAV envelope glycoprotein. *Nature (Lond.),* **323,** 725.

Ljunggren K., Böttiger B., Biberfeld G., Karlsson A., Fenyö E. M. & Jondal M. (1987) Antibody dependent cellular cytotoxicity-inducing antibodies against human immunodeficiency virus: presence at different clinical stages. *J. Immunol.* **139,** 2263.

Modrow S., Hahn B.H., Shaw G.M., Gallo R.C., Wong-Staal F. & Wolf H. (1987) Computer-assisted analysis of envelope protein sequences of seven human immunodeficiency virus isolates: Prediction of antigenic epitopes in conserved and variable regions. *J. Virol.* **61,** 570.

Muesing M.A., Smith D.H., Cabradilla C.D., Benton C.V., Lasky L.A. & Capon D.J. (1985) Nucleic acid structure and expression of the human AIDS/lymphadenopathy retrovirus. *Nature (Lond.)* **313,** 450.

MYERS G., JOSEPHS S.F., RABSON A.B., SMITH T.F. & WONG-STAAL F. (1988) *Database Human Retroviruses and AIDS.* Los Alamos Natl. Lab., Los Alamos.

NÄRVÄNEN A., KORKOLAINEN M., KONTIO S., SUNI J., TURTIANEN S., PARTANEN P., SOOS J., VAHERI A. & HUHTALA M.-L. (1988) Highly immunoreactive antigenic site in a hydrophobic domain of HIV-1 gp41 which remains undetectable with conventional immunochemical methods. *AIDS*, **2,** 119.

NEOH S.H., GORDON C., POTTER A. & ZOLA H. (1986) The purification of mouse monoclonal antibodies from ascites fluid. *J. immunol. Meth.* **91,** 231.

NIEDRIG M., HINKULA J., WEIGELT W., L'AGE-STEHR J., PAULI G., ROSEN J. & WAHREN B. (1989) Epitope mapping of monoclonal antibodies against human immunodeficiency virus type 1 structural proteins by using peptides. *J. Virol.* **63,** 3525.

OHLIN M., DANIELSSON L., CARLSSON R. & BORREBAECK C.A.K. (1989) Effect of leucyl-leucine methyl ester on proliferation and Ig secretion of EBV-transformed human B lymphocytes. *Immunology* **66,** 785.

PALKER T.J., CLARK M.E., LANGLOIS A.J., MATTHEWS T.J., WEINHOLD K.J., RANDALL R.R., BOLOGNESI D.P. & HAYNES B.F. (1988) Type-specific neutralization of the human immunodeficiency virus with antibodies to env-encoded synthetic peptides. *Proc. natl. Acad. Sci. U.S.A.* **85,** 1932.

PRINCE A.M., HOROWITZ B., BAKER L., SCHULMAN R.W., RALPH H., VALINSKY J. *et al.* (1988) Failure of a human immunodeficiency virus (HIV) immune globulin to protect chimpanzees against experimental challenge with HIV. *Proc. natl. Acad. Sci. U.S.A.* **85,** 6944.

PUTNEY S.D., MATTHEWS T.J., ROBEY W.G., LYNN D.L., ROBERT-GUROFF M., MUELLER W.T. *et al.* (1986) HTLV-III/LAV. Neutralizing antibodies to an *E. coli*-produced fragment of the virus envelope. *Science*, **234,** 1392.

RATNER L., HASELTINE W., PATARCA R., LIVAK K.J., STARCICH B., JOSEPHS S.F. *et al.* (1985) Complete nucleotide sequence of the AIDS virus, HTLV-III *Nature (Lond.)*, **313,** 277.

REICHMANN L., CLARK M., WALDMANN H. & WINTER G. (1988) Reshaping human antibodies for therapy. *Nature (Lond.)*, **332,** 323.

RUSCHE J.R., JAVAHERIAN K., MCDANAL C., PETRO J., LYNN D.L., GRIMAILA R. *et al.* (1988) Antibodies that inhibit fusion of human immunodeficiency virus-infected cells bind a 24-amino acid sequence of the viral envelope, gp120. *Proc. natl. Acad. Sci. U.S.A.* **85,** 3198.

SARNGADHARAN M.G., POPOVIC M., BRUCH L., SCHUPBACH J. & GALLO R.C. (1984) Antibodies reactive with human T-lymphotropic retroviruses (HTLV-III) in the serum of patients with AIDS. *Science*, **224,** 506.

SCHRIER R.D., GNANN J.W. JR., LANGLOIS A.J., SHRIVER K., NELSON J.A. & OLDSTONE M.B.A. (1988) B- and T-lymphocyte response to an immunodominant epitope of human immunodeficiency virus. *J. Virol.* **62,** 2531.

SMITH R.S., NASO R.B., ROSEN J., WHALLEY A., HOM Y.-L., HOEY K., KENNEDY C.J., MCCUTCHAN J.A., SPECTOR S.A. & RICHMAN D.D. (1987) Antibody to a synthetic aligopeptide in subjects at risk for human immunodeficiency virus infection. *J. clin. Microbiol.* **25,** 1498.

WAHREN B., CHIODI F., LJUNGGREN K., PUTTNEY S., KURTH R., GALLO R.C. & FENYÖ E.M. (1988) B and T cell reactivities after immunization of macaques with HIV subcomponents. *AIDS Res. Human Retroviruses*, **4,** 199.

WANG J.J.G., STEEL S., WISNIEWOLSKI R. & WANG C.Y. (1986) Detection of antibodies to human T-lymphotropic virus type III by using a synthetic peptide of 21 amino acid residues corresponding to a highly antigenic segment og gp41 envelope protein. *Proc. natl. Acad. Sci. U.S.A.* **83,** 6159.

WEBER J.N., CLAPHAM P.R., WEISS R.A., PARKER D., ROBERTS C., DUNCAN J., WELLER I., CARNE C., TEDDER R.S., PINCHING A.J. & CHEINSONG-POPOV R. (1987) Human immunodeficiency virus infection in two cohorts of homosexual men; neutralizing sera and association of anti-gag antibody with prognosis. *Lancet*, **i,** 119.

WEISS R.A., CLAPHAM P.R., CHEINSONG-POPOV R., DALGLEISH A. G., CARNE C.A., WELLER I.V.D. & TEDDER R.S. (1985) Neutralization of human T-lymphotropic virus type III by sera of AIDS and AIDS-risk patients. *Nature (Lond.)*, **316,** 69.

WEISS R.A., CLAPHAM P.R., WEBER J.N., DALGLEISH A.G., LASKY L.A. & BERMAN P.W. (1986) Variable and conserved neutralization antigens of human immunodeficiency virus. *Nature (Lond.)*, **324,** 572.

WONG-STAAL F. & GALLO R.C. (1985) Human T-lymphotropic retroviruses. *Nature (Lond.)*, **317,** 395.