Journal of Immunological Methods, 118 (1989) 1–7
Elsevier

JIM 05077

Carrier-bound synthetic peptides

Use as antigen in HIV-1 ELISA tests and in antiserum production

Susanne Modrow [1], Brigitte Höflacher [2], Ronald Mertz [3] and Hans Wolf [1]

[1] *Max von Pettenkofer Institute, Pettenkoferstr. 9a, 8000 Munich 2, F.R.G.,* [2] *Gesellschaft für Strahlen- und Umweltforschung, Ingolstädter Landstrasse 1, 8042 Neuherberg, F.R.G., and* [3] *Genzentrum, Am Klopferspitz 18a, 8033 Martinsried, F.R.G.*

(Received 30 June 1988, revised received 3 October 1988, accepted 17 October 1988)

Chemically synthesized carrier-bound peptides have been used as antigens in diagnostic test systems (ELISA) and for raising antipeptide-specific antisera. The method does not require prior cleavage of the peptides from the support used for the solid-phase synthesis. Using the same resin for both the synthesis and the subsequent applications it was possible to avoid expensive and time-consuming purification procedures and artificial recoupling to solid supports. A quick and specific ELISA-based diagnostic test system for HIV-specific antipeptide antibodies in human sera was established. In addition the carrier-bound peptides were shown to be potent antigens for raising antibodies in animals.

Key words: Synthetic peptide; ELISA; HIV-1

Introduction

Since solid-phase peptide synthesis was introduced (Merrifield, 1963) the method has been established in many laboratories and the use of synthetic peptides has become widespread in many areas of the natural and medical sciences. In particular, synthetic peptides were introduced as specific antigens in diagnostic test systems for the detection of antibodies in pathological sera (Neurath et al., 1982; Modrow and Wolf, 1986). Monospecific (Lerner, 1982, Dietzschold et al., 1984; Kris et al., 1985) or monoclonal (Scheefers-Borchel et al., 1985) antisera against peptide sequences have been produced and used for the characterization and identification of native polypeptides in cellular and molecular systems and have proven to be of value in immunological test systems. With the expanding use of oligopeptides, the chemical methods for synthesis have been considerably improved and adapted to automated systems which allow synthesis of peptide sequences in a relatively short time with high yields. The consecutive purification steps, however, are still very expensive and time-consuming and require highly developed technical equipment for the cleavage of side-chain protection groups and HPLC column systems for purification. Peptides used in diagnostic tests such as ELISA (enzyme-linked immunosorbent assay) or RIA (radioimmunoassay) have to be coupled to a solid-plastic support; in a similar manner coupling to larger proteins, i.e., KLH (keyhole limpet hemocyanin) or BSA (bovine serum albumin), is necessary for antibody production. However, in many cases these coupling procedures cause the destruction of secondary structure so that the peptide antigens no longer correspond to the na-

Correspondence to: H. Wolf, Max von Pettenkofer-Institut, Ludwig-Maximilians-Universität, Pettenkoferstr. 9a, D-8000 Munich 2, F.R.G.

tive form. To avoid these problems we have developed a system in which side-chain protection groups are removed without the simultaneous cleavage of the synthesized peptide from the polystyrene support. The polystyrene beads have a large active surface and can be directly used in specific and sensitive diagnostic tests and to raise antibodies in rabbits or other animals.

Materials and methods

Solid support

A copolymer of styrene with 1% divinylbenzene (= 1% cross-linked polystyrene) with a diameter of 20–50 μm was used as the solid support for peptide synthesis. The benzylchloride groups of the resin were esterified with *N-t*-Boc-alanine (*N-t*-butoxycarbonyl-alanine) as the first amino acid of the peptide sequence in a substitution of 0.59 mmol alanine/g resin (the resin was purchased from Bachem, Bubendorf, Switzerland). The *N-t*-Boc group was removed by treatment with 25% trifluoroacetic acid in dichloromethane for 30 min followed by a series of three washes with dichloromethane, absolute ethanol and dichloromethane. The resin was dried and an equivalent of 0.5 mmol alanine was used for peptide synthesis.

Seclection of peptide sequences

HIV-1-specific proteins with diagnostic relevance (p17, p24, gp41) derived from the strain BH10/HTLV-III (Ratner et al., 1985) were first analyzed in order to identify potential antigenic sites. This analysis was done by using a computer program written for a VAX 750 (Wolf et al., 1988) which calculates secondary structures according to Chou and Fasman (1987) or Garnier et al. (1978) and superimposes those predictions on the values for local hydrophilicity (Hopp and Woods, 1981) and surface probability (Emini et al., 1985) or flexibility (Karplus and Schulz, 1985). Peptide sequences with a high content of β-turns in a hydrophilic environment, with high values for surface probability and flexibility, were interpreted as likely candidates for antigenic sites since they frequently form loop-like structures at the protein surface. According to this analysis the following sequences were selected for synthesis: (i) amino acids 109–123 of the *gag* protein complex: NH_2-Asn-Lys-Ser-Lys-Lys-Lys-Ala-Gln-Gln-Ala-Ala-Ala-Asp-Thr-Gly-Ala; this sequence is part of the p17 of the *gag* complex; (ii) amino acids 226–237 of the *gag* protein complex of HIV-1 (HTLV-III/BH10); NH_2-Gly-Gln-Met-Arg-Glu-Pro-Arg-Gly-Ser-Asp-Ile-Ala; this sequence is part of p24 of the *gag* protein complex; (iii) amino acids 655–667 of gp41 of HIV-1 (HTLV-III/BH10): NH_2-Glu-Ser-Gln-Asn-Gln-Gln-Glu-Lys-Asn-Glu-Gln-Glu-Leu-Ala.

Peptide synthesis

Peptides were synthesized in a 430A peptide synthesizer (Applied Biosystems, Weiterstadt, F.R.G.) using Fmoc (9-fluorenylmethyloxycarbonyl)-protected amino acids (Atherton et al., 1978); in order to complete the coupling reactions double-couple cycles were used for each amino acid. *t*-butyl-(Ser, Asp), *t*-butoxycarbonyl-(Lys) and 4-methoxy-2,3,6-trimethylbenzenesulfonyl (Arg) groups were used for side-chain protection. Fmoc-protected amino acids were converted to hydroxybenzotriazol-activated esters by treatment with 1.5 mmol hydroxybenzotriazol and 1.2 mmol diisopropylcarbodiimide/mmol amino acid for 30 min in dimethylformamide; the subsequent coupling reaction was performed in dimethylformamide. After the coupling reaction, Fmoc groups were removed with 20% piperidine in dimethylformamide followed by a series of washes with dimethylformamide. All of the amino acids used were purchased from Bachem, Bubendorf, Switzerland, chemicals were obtained from Aldrich, Steinheim, F.R.G. and the solvents were from Merck, Darmstadt, F.R.G. After synthesis side-chain protecting groups were removed in TFA with 10% thioanisol and 10% *m*-cresol as scavengers. The resin-bound peptides were washed in series with an excess of dichloromethane, ethyldiisopropylamide and dichloromethane and dried. All reaction steps are shown in Fig. 1. For confirmation of the amino acid sequence, the carrier-bound peptides were sequenced using an Applied Biosystems gas phase sequencer.

ELISA tests

The ELISA tests were conducted in a Millipore filtration system using 96-well plates sealed on the

bottom with hydrophilic membrane (GV) (pore diameter 0.2 μm), which has a very low protein binding capacity (Millititersystem, Millipore, Eschborn, F.R.G.). The resin-bound peptides were suspended in PBS containing 0.1% Tween 20 and differing amounts (10 ng to 10 μg) of resin-bound peptide were added to the wells. The beads were washed four times with 100 μl PBS/0.1% Tween 20 and incubated 1 h in a solution of 5 mg gelatine in H_2O and washed again. HIV-1-positive and -negative human sera were diluted in PBS/0.1% Tween 20/1% rabbit serum. 100 μl of each dilution were added and incubated with the resin-bound peptide for 1 h. The beads were washed four times with PBS/0.1% Tween 20, incubated with rabbit anti-human IgG (Dako, diluted 1/1000 in PBS/0.1% Tween 20) for an additional hour followed by four washes in PBS/0.1% Tween 20. For color development, 100 μl phosphate buffer pH 6.0 containing 0.5 mg/ml *o*-phenylenediamine and 0.1% H_2O_2 were added for 2 min. The solution was then transferred to a 96-well ELISA plate (Dynatech), each well containing 100 μl 1 M H_2SO_4 to stop the color reaction. Optical densities were determined at 486 nm. In order to demonstrate peptide-antibody reaction, p226–237/p24 beads were incubated with HIV-positive serum (diluted 1/500) and ferritin or peroxidase-labeled rabbit anti-human IgG. The beads with the bound complexes were examined by electron microscopy.

Production of antipeptide sera

For the production of antisera, rabbits were inoculated subcutaneously with 1 ml of an emulsion of 500 μg resin-bound peptide of 226–237/p24-HIV-1 with and without complete Freund's adjuvant in PBS. Booster injections were given at intervals of 4 weeks with and without incomplete Freund's adjuvant. Serum was taken 1 week after the booster injections in each case and tested for antipeptide-specific antibodies in ELISA tests. In addition, the specificity of the rabbit sera was tested on Western blots for reactivity with p24 of HIV-1. HIV-1-infected H9 cells were solubilized and the proteins submitted to SDS-PAGE and Western blotting as described (Gürtler et al., 1987).

Results

Peptide synthesis

Using chloromethylated polystyrene with the first amino acid bound in ester linkage to the resin, peptides were synthesized using orthogonal solid phase peptide synthesis with Fmoc-protected amino acids (Fig. 1). In order to stabilize the linkage to the resin for the treatment with secondary amine to remove the Fmoc protection group and with trifluoroacetic acid to cleave the side-chain protection groups, amino acids with strong electron-donating (Phe, Leu) or electron-attracting (Arg, Lys) functions were avoided. In these experiments we selected alanine as the first

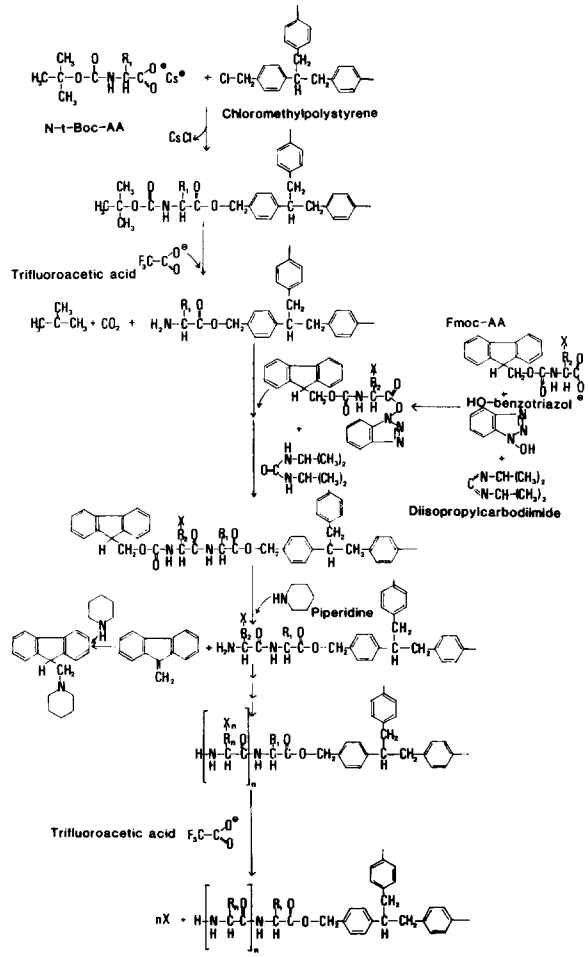

Fig. 1. Reaction steps used for the synthesis of carrier-bound peptides. X, permanent side chain protection group. R_1, R_2, R_n, amino acid residues.

amino acid with no functional side-chain which should have given an approximately equal distribution of electron density in the linkage group. The synthesized peptides were sequenced and found to have the correct amino acid sequences. From this sequencing data it could be concluded that the material was 90% pure (M. Eulitz, GSF Munich, personal communication). Since no further purification procedures were used, this degree of homogeneity was probably due to the double coupling of each residue and (with respect to HF or TFMSA cleavage) to the relatively mild treatment with diluted trifluoroacetic acid to remove the acid-labile side-chain protection groups.

ELISA test systems

The Millititer filtration system, with a hydrophilic Durapore membrane which was used for the ELISA tests was characterized by a very low protein binding capacity. In order to block residual protein binding to the plastic wells or to the resin, both were incubated with a gelatin solution before the addition of the antisera. The amount of antigen necessary to obtain a positive signal was titrated using a positive human HIV-I serum pool and positive signals were still observed with 10 ng beads/well (= ca. 5 ng of peptide). Because of the high substitution of beads with peptide, further tests were done with 1 μg beads (= ca. 500 ng of peptide) per well since at lower concentrations a statistically equal distribution of beads per well could not be ensured.

In addition to the small amounts of specific peptide antigen sufficient for a positive reaction, the ELISA test using carrier-bound peptides 'in solution' could be completed in a very short time (~ 3 h); this was probably due to the optimal antigen presentation of the peptides on the surface of the carrier. For comparison, p655–667/gp41 was synthesized using *t*-Boc chemistry and the peptide was cleaved with HF, purified and then coupled to ELISA plates in the traditional manner using sodium cabonate buffer (pH 9.5). Those tests showed a higher background and were found to be less sensitive and less specific.

To demonstrate antigen-antibody complex formation on the surface of the beads, ferritin-labeled anti-human IgG was used as the second antibody and the beads were examined by electron microscopy. Ferritin grains were observed to be localized in very dense concentrations on the carrier surface and no grains could be seen inside the

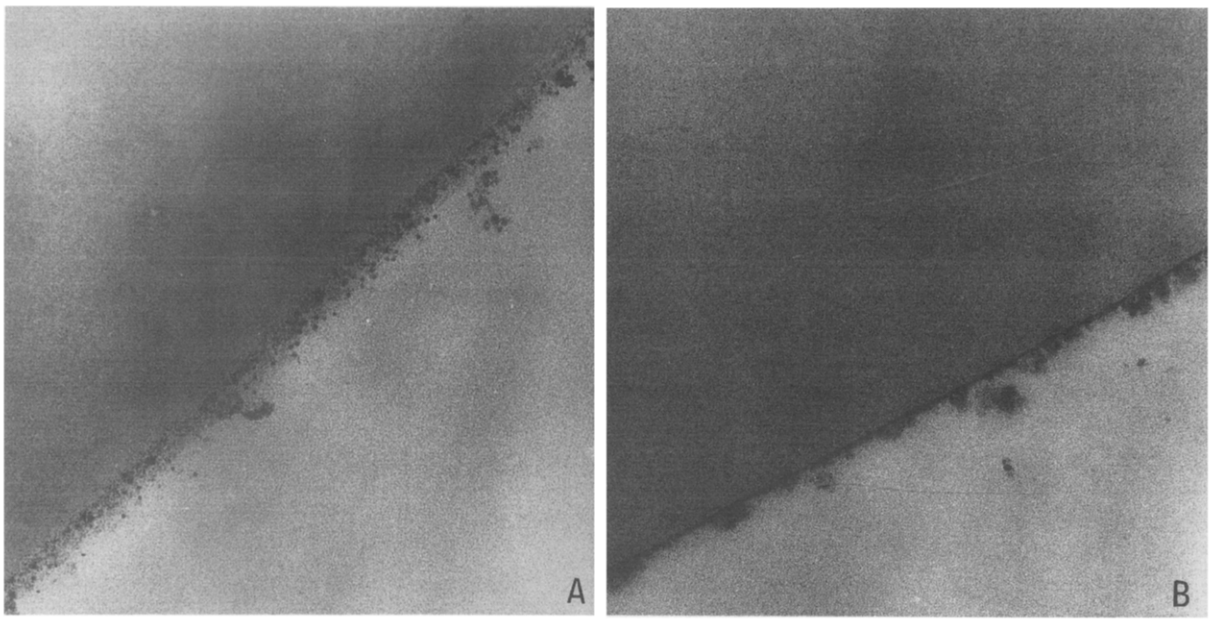

Fig. 2. Electronmicroscopy (×100000) of peptide beads 226–237/p24 following reaction with an HIV-positive serum. *A*: using ferritin-labeled anti-human IgG as second antibody. *B*: using peroxidase-labeled anti-human IgG as second antibody.

TABLE I

COMPARISON OF THE RESULTS OBTAINED WITH VARIOUS HIV-I-POSITIVE SERA IN AN ELISA USING CARRIER-BOUND PEPTIDE AND IN WESTERN BLOT EXPERIMENTS.

Serum	Peptide-ELISA			Western-blot			Comments
	p109–123/p17	p226–237/p24	p655–667/gp41	p17	p24	gp41	
1	1/200	1/200	1/400	+	++	+	
2	–	1/200	1/200	–	+	+	
3	1/400	1/200	1/100	++	++	(+)	Early infection
4	–	1/400	1/200	–	++	+	
5	1/200	1/400	1/800	+	(+)	++	AIDS
6	1/200	1/400	1/400	+	++	++	
7	1/800	1/1600	1/400	++	++	+	
8	–	1/100	1/1600	–	(+)	++	AIDS
9	1/200	1/400	1/800	+	++	++	
10	1/200	1/400	1/1600	+	+	+++	
11	1/100	1/400	1/400	+	++	++	
12	1/100	1/400	1/200	+	+	+	
13	–	1/200	1/400	(+)	+	++	
14	–	1/400	1/800	–	++	++	
15	1/200	1/800	1/400	++	++	+	

beads (Fig. 2). This may have been due to the relatively large size of the ferritin-labeled antibody molecule. Using peroxidase-labeled anti-human IgG, the reaction appeared, in part, to be internal. HIV-1 positive sera taken from patients at different stages of the viral infection were titrated up to dilutions of 1/10000. Positive signals were obtained in the dilution range of 1/120 to 1/5000 for the respective sera. The values of each individual serum were compared with the results obtained from continuously used Western blot tests of HIV-1 positive sera from electrophoretically separated viral antigens; values for the ELISA test were found to coincide closely with the results from Western blots (Table 1). HIV-negative sera showed, in some cases, a background reaction up to dilutions of 1/40.

Production of peptide-specific antisera

Rabbits were inoculated with carrier-bound peptide p226–237/p24 alone or emulsified with Freund's adjuvant. Sera were taken 1 week after the booster injections and tested for anti-peptide-specific antibodies (Table II). The rabbits, inoculated with peptide-antigen and Freund's adjuvant developed antibodies after the first booster inoculation and showed higher anti-peptide titers. The sera were tested for their reactivity with the native antigen on Western blots; for these experiments HIV-1 proteins were separated by SDS-PAGE and transferred to nitrocellulose. The p24-specific band could be identified in all sera. For further confirmation of the specificity of the antibody reaction, purified p24 produced by recombinant gene technology in bacteria (E. Soutschek-Bauer, M. Motz, personal communication) was coupled to ELISA plates. The sera of the rabbit inoculated with peptide beads in combination with Freund's adjuvant showed positive reactions in

TABLE II

ANTI-PEPTIDE TITERS OBTAINED IN RABBITS AFTER INOCULATION WITH CARRIER-BOUND PEPTIDE 226–237/p24. SERUM WAS TAKEN 1 WEEK AFTER BOOSTER INJECTIONS IN EACH CASE AND TESTED FOR ANTI-PEPTIDE SPECIFIC ANTIBODIES.

Time (weeks after inoculation)	Rabbit 1 carrier-bound peptide	Rabbit 2 carrier-bound peptide in combination with Freund's adjuvant
0	–	–
5	1/16	1/32
9	1/32	1/64
13	1/160	1/320
17	1/640	1/1280
21	1/1280	1/2560

dilutions up to 1/1000; sera from the rabbit immunized without Freund's adjuvant could be titrated up to 1/500.

Discussion

Using a combination of 1% cross-linked polystyrene resin with the first amino acid complex in ester linkage to the chlormethylated benzyl groups (as generally used in *N-t*-Boc peptide chemistry) and the addition of Fmoc-protected amino acids, it was possible to obtain pure peptides still connected to their solid support and with all side-chain blocking groups removed. Cleavage from the resin of peptides bound as benzyl esters has been shown to occur after treatment with 1% trifluoroacetic acid (Stewart and Young, 1984). However, in the present method the resin was heavily substituted with alanine (0.59 mmol/g resin) as the first amino acid and the trifluoroacetic acid treatments did not result in a substantial loss of peptide from the resin. Similarly, the peptide-resin linkage was resistent to treatment with secondary amine to remove Fmoc groups after each coupling step. In each case the sequences of the synthesized peptides were confirmed by amino acid analysis.

After extensive washing of the resin-bound peptide it was possible to use the peptides without any further purification steps in diagnostic ELISA tests for screening human sera for HIV-specific antibodies. In these test systems the antigens were presented in a way which may mimic their structure in the native proteins. It is known that protein-binding to plastic supports, which is done routinely at high pH values (pH 9.5), destroys the secondary structure of many antigens. This may be avoided by an ELISA test in solution with the antigens bound to the solid support at their carboxy-terminal ends. In addition to the optimal antigen presentation in solution which permitted a reduction of the antigen concentration to 5 ng peptide per well, it was possible to shorten all the incubation times and the total test could be performed in about 3 h. Furthermore, the antibody complexes could be removed from the peptide beads by treatment with detergent and re-used.

In addition, we have shown that it is possible to raise specific antipeptide sera in rabbits without any further purification or coupling to other protein material known to destroy most of the secondary structure antigens. Polystyrene is a rather weak antigen and specific antipeptide sera were readily obtained after inoculation.

The method described here is inexpensive and should permit rapid, simple diagnostic test systems to be established for a wide range of infectious diseases.

Acknowledgements

This work was supported by the Fond der Chemischen Industrie, F.R.G.

The authors wish to thank M. Eulitz (GSF, Munich) for peptide sequencing, H. Gelderblom (BGA, Berlin) for electronmicroscopy, L. Gürtler (Max von Pettenkofer Institute, Munich) for HIV-1-positive sera and B. Dietzschold (Wistar Institute, Philadelphia, PA) for helpful discussions.

References

Atherton, E., Fox, H., Logan, C.J., Harkiss, D., Sheppard, R.C. and Williams, B.J. (1978) A mild procedure for solid phase peptide synthesis. Use of fluorenylmethyloxycarbonyl amino acids. J. Chem. Soc. Chem. Commun. 13, 539–540.

Chou, P.Y. and Fasman, G.D. (1978) Prediction of the secondary structure of proteins from their amino acid sequence. Adv. Enzymol. 47, 45–148.

Dietzschold, B., Eisenberg, R.J., Ponce de Leon, M., Golub, E., Hudecz, F., Varrichio, A. and Cohen, G.H. (1984) Fine structure analysis of type-specific and type-common antigenic sites of herpes simplex virus glycoprotein D. J. Virol. 52, 431–435.

Emini, E.A., Hughes, J.V., Perlow, D.S. and Beger, D. (1985) Induction of hepatitis A virus neutralizing antibody by a virus-specific synthetic peptide. J. Virol. 55, 836–839.

Garnier, J., Osguthorpe, O.J. and Robson, B. (1978) Analysis of the accuracy and implication of simple methods for predicting the secondary structure of globular proteins. J. Mol. Biol. 120, 97–120.

Gürtler, L., Eberle, J., Lorbeer, B. and Deinhardt, F. (1987) Sensitivity and specificity of commercial ELISA kits for screening the LAV/HTLV-III. J. Virol. Methods 15, 11–23.

Hopp, T.P. and Woods, K.P. (1981) Prediction of protein antigen determinants from amino acid sequences. Proc. Natl. Acad. Sci. U.S.A. 78, 3824–3828.

Karplus, P.A. and Schulz, G.E. (1985) Prediction of chain flexibility in proteins. Naturwissenschaften 72, 212–213.

Kris, R.M., Lax, I., Gullick, W., Waterfield, M.D., Ullrich, A., Fridkin, M. and Schlesinger, I. (1985) Antibodies against a synthetic peptide as a probe for the kinase activity of the avian EGF receptor and v-erb B protein. Cell 40, 619–625.

Lerner, R.A. (1982) Mapping the immunological repertoire to produce antibodies of predetermined specificity. Nature 299, 592–596.

Merrifield, R.B. (1963) Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. J. Am. Chem. Soc. 85, 2149–2154.

Modrow, S. and Wolf, H. (1986) Characterization of two related Epstein-Barr virus-encoded membrane proteins that are differentially expressed in Burkitt lymphoma and in vitro-transformed cell lines. Proc. Natl. Acad. Sci. U.S.A. 83, 5703–5707.

Neurath, A.R., Kent, S.B.H. and Strick, N. (1982) Specificity of antibodies elicited by a synthetic peptide having a sequence in common with a fragment of a virus protein, the hepatitis B surface antigen. Proc. Natl. Acad. Sci. U.S.A. 79, 7871–7875.

Ratner, L., Haseltine, W., Patarca, R., Livak, K.J., Starcich, B., Josephs, S.F., Doran, E.R., Ratalski, J.A., Whitshoon, E.A., Baumeister, K., Ivanoff, L., Petteway, S.R., Pearson, M.L., Lautenberger, I.A., Papas, T., Ghrayeb, I., Chang, N.T., Gallo, R.C. and Wong-Staal, F. (1985) Complete nucleotide sequence of the AIDS virus HTLV-III. Nature 313, 277–284.

Scheefers-Borchel, U., Müller-Berghans, G., Fuhge, P., Eberle, R. and Heimburger, N. (1985) Discrimination between fibrin and fibrinogen by a monoclonal antibody against a synthetic peptide. Proc. Natl. Acad. Sci. U.S.A. 82, 7091–7095.

Stewart, J.M. and Young, J.D. (9184) Solid Phase Peptide Synthesis. Freeman, San Francisco, CA.

Wolf, H., Modrow, S., Motz, M., Jameson, B.A., Hermann, G. and Förtsch, B. (1988) An integrated family of amino acid sequence analysis programs. Comput. Appl. Biol. Sci. 4, 187–191.