most aflatoxins tested. The concentrations causing 50% inhibition of binding of $^3$H-$AFB_1$ (values in the parentheses indicate relative cross-reactivity), to the anti-$AFB_3$-HS-BSA antibody by unlabeled $AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$, and $AFB_3$ are found to be 0.25 (*100*), 3.34 (*7.5*), 0.32 (*78.1*), 4.0 (*6*), and 0.53 (*47.2*) ng/assay, respectively. From the displacement curve, it is apparent that the range for the detection $AFB_1$ and $AFG_1$ of the present RIA system falls between 1 and 100 ng/ml (or 0.05–5 ng/assay). Such sensitivity is within the range of most other RIA systems (0.5–5.0 ng/assay) for aflatoxins [5, 6]. Since the antibodies elicited have almost equal cross-reactivity with both $B_1$ and $G_1$, such antibodies would be very useful for simultaneous detection of both toxins. The low cross-reactivity of the antibodies with $AFB_2$ and $AFG_2$ would not limit the wide use of these antibodies for aflatoxin assay because these two aflatoxins rarely occur in agricultural commodities. Also, the toxicity and carcinogenicity of these two aflatoxins are relatively low. Since tritiated $AFB_1$ was used as the marker ligand in the RIA, the apparent cross-reactivity of the antibodies with $AFB_3$ was slightly lower than for $AFB_1$ and $AFG_1$. In view of the importance of use of this antibody for immunoassay of aflatoxins, studies such as the development of an ELISA method for simultaneous detection of both aflatoxins as well as using this new immunogen for generating monoclonal antibody for aflatoxins are currently underway in our laboratory.

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1 Busby, W. F. Jr, and Wogan, G. N., in: Food-borne Infections and Intoxications, 2nd edn, p. 519. Eds H. Riemann and F. L. Bryan. Academic Press, New York/London 1979.
2 Chu, F. S., J. Fd Prot. *47* (1984) 562.
3 Chu, F. S., in: Mycotoxins and Phytotoxins, p. 277. Eds P. S. Steyn, and R. Vleggaar. Elsevier, Amsterdam 1986.
4 Chu, F. S., in: Modern Methods in the Analysis and Structural Elucidation of Mycotoxins, p. 207. Ed. R. J. Cole. Academic Press, New York 1986.
5 Chu, F. S., and Ueno, I., Appl. envir. Microbiol. *33* (1977) 1125.
6 Chu, F. S., Lee, R. C., Trucksess, M. W., and Park, D. L., J. Ass. off. analyt. Chem. *71* (1988).
7 Wei, R. D., and Chu, F. S., Analyt. Biochem. *160* (1987) 399.
8 Chu, F. S., J. Ass. off. analyt. Chem. *54* (1971) 1304.
9 Heathcote, J. G., and Dutton, M. F., Tetrahedron *25* (1969) 1497.
10 Cole, R. J., and Kirksey, J. W., Agric. Fd Chem. *19* (1971) 222.
11 Chu, F. S., Grossman, S., Ru-Dong Wei, and Mirocha, C. J., Appl. envir. Microbiol. *37* (1979) 104.
12 Habeeb, A. F. S. A., Analyt. Biochem. *14* (1966) 326.
13 Harder, W. O., and Chu, F. S., Experientia *35* (1979) 1104.
14 Gaur, P. K., Lau, H. P., Pestka, J. J., and Chu, F. S., Appl. envir. Microbiol. *41* (1981) 478.
15 Sizaret, P., Malaveille, C., Montesano, R., and Frayssinet, C., J. natl Cancer Inst. *69* (1980) 1375.

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## Profile of sequential determinants in tissue polypeptide antigen BrCN:B fragment

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*Summary.* A synthetic approach has been applied to determine the profile of sequential determinants of one immunodominant region of *T*issue *P*olypeptide *A*ntigen (TPA). Five overlapping peptides, covering 30 of the 32 amino acid residues of this fragment, were chemically synthesized, and their antibody-binding activities for rabbit anti-TPA antibodies determined by enzyme-linked immunoadsorbant assays.
Anti-TPA reacted with two overlapping fragments at the COOH-terminal end of the fragment, but not with peptides that include Arg 15 considered as essential for the antigenicity of the whole fragment. This might suggest that this critical residue is involved in the formation of a complex conformational determinant.
*Key words.* TPA; synthetic peptides; sequential determinants.

The *T*issue *P*olypeptide *A*ntigen (TPA), discovered in 1957 by Bjorklund [1] has been demonstrated by several authors to be a potential marker in different neoplasias [2]; this protein is apparently produced and released by cancer cells, and its level in serum is correlated significantly with the progression of the neoplastic disease [3].
Because of the role that TPA plays in cancer detection and diagnosis, there has been much interest in isolating this protein in a pure form, and in defining its biochemical and biophysical properties. Also, since TPA is detected and quantitated in biological fluids by the aid of anti-TPA sera, much effort has been dedicated to the identification of the immunogenic regions of this antigen.
In aqueous solution, TPA has the tendency to form high molecular weight aggregates, that can be dissociated by SDS into subunits. The B1 subunit (mol.wt 43,000) has been partially characterized from the biochemical point of view, and several cyanogen bromide fragments have been isolated and sequenced [4]. TPA fragment BrCN:B, a 32-residue-long peptide that shares over 70% homology with sequence 56–87 of human epidermal 50 K keratin, seems to include at least one antigenic determinant, since anti-TPA sera react with synthetic peptides that closely resemble in sequence this cyanogen-bromide fragment [5]. The exact location and nature of the epitope(s) is unknown, although preliminary investigations indicate that Arg 15 is essential for the binding of anti-TPA antibodies, and that one antigenic site should be therefore located around this amino acid. In this investigation, we examined the profile of continuous antigenic determinants recognized by commercial anti-TPA sera in this immunogenic 32-residue fragment. Five synthetic peptides, that were from 9- to 15-residues-long and covered the full length of the BrCN:B fragment, were chemically synthesized by the solid-phase method [6] using a DuPont-Vega Coupler

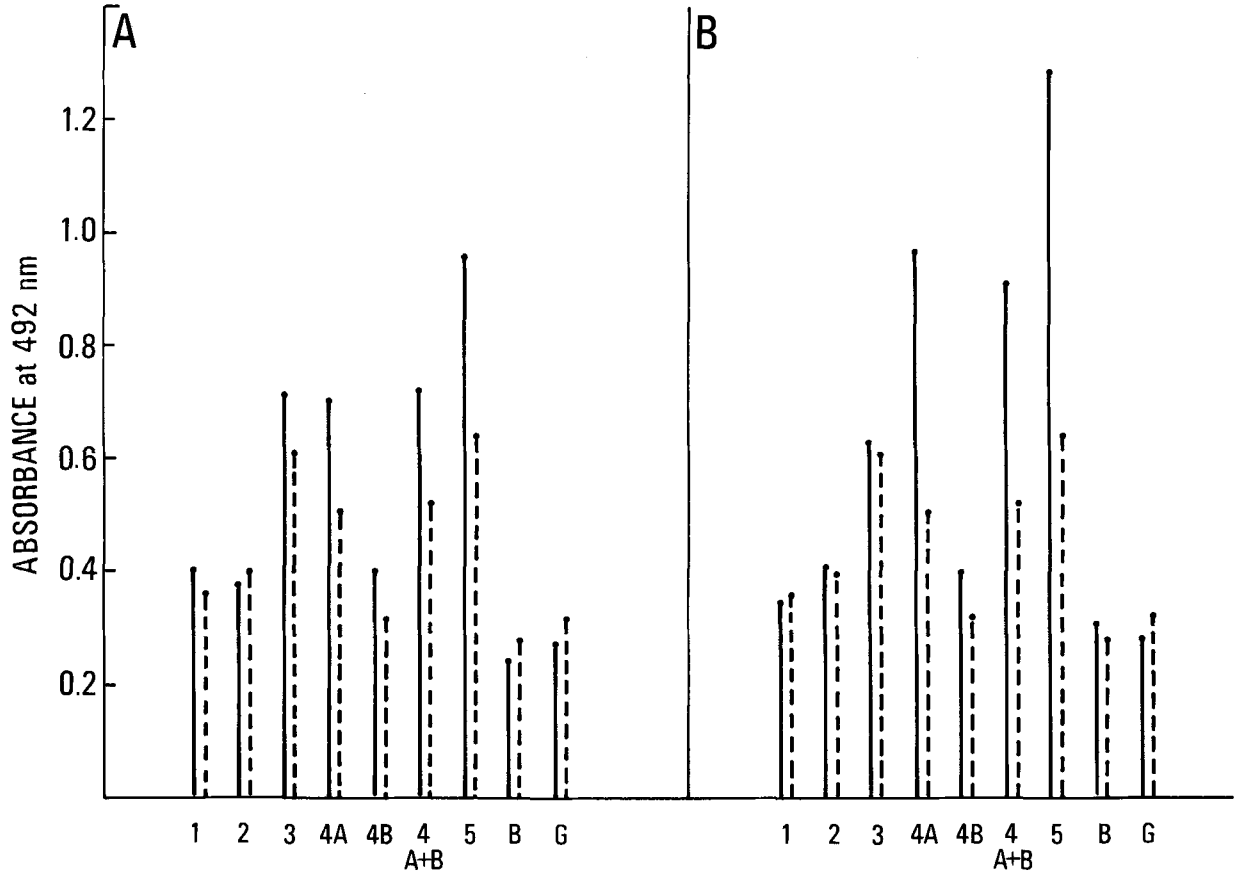
Mod. 1000. Each synthesis was initiated with approx. 1 g of Boc-amino acid-PAM resin and in conjunction with the following sidechain protecting group: O-benzyl (glutamic acid, aspartic acid), tosyl (arginine), O-bromo-benzyloxycarbonyl (tyrosine). The couplings were carried out with a 4-fold excess of protected amino acid and a 6-fold excess of dicyclohexylcarbodiimide. Most couplings were over 95% complete as determined by the ninhydrin test. Second couplings were used when the first resulted unsatisfactorily. The final protected peptides were treated with trifluoromethansulfonic acid (TFMSA) at 45° C for 45 min [7], then isolated by gel filtration on a Sephadex G 25 superfine column in 0.01 M ammonia. Peptide 4 yielded a broad peak, that was tentatively pooled into two fractions, A and B, corresponding to the first and second halves of the peak, respectively. The amino acid analysis indicated that all peptides except 4 B had compositions that were ±5% of the theoretical value. 4 B proved to be very heterogeneous, as revealed by ion exchange column chromatography on a Dowex 50 × 2 column equilibrated and developed with pyridin-acetic acid buffers (data not shown).

The amino acid sequence of TPA BrCN:B fragment and the location of the five fragments selected for synthesis are reported in the table. The hydrophilicity index (H.I.) for the

Amino acid sequence of TPA BrCN:B fragment aligned with human epidermal 50 K keratin, and location of the five synthetic peptides used for binding assays

| | | | |
|---|---|---|---|
| I) | | A V L N D R L A Q Y L D E V R A L E A A N G R L E V L D E L N L | |
| II) | | Q N L N D R L A S Y L D K V R A L E E A N A D L E V K I R D W – | |
| | | | H. I. |
| 1 | 1–10 | | – 0.20 |
| 2 | 7–18 | | + 0.16 |
| 3 | 12–22 | | + 0.67 |
| 4 | 16–30 | | + 0.33 |
| 5 | 22–30 | | + 0.56 |

I) TPA BrCN:B fragment; II) human epidermal 50 K keratin.




*A* Binding of IgG isolated from commercial anti-TPA:B1 by chromatography on DEAE Affi-gel Blue, to the synthetic peptides, as derived from ELISA (enzyme-linked immuno-sorbant assay). Wells of microtiter plates were coated with 1 µg of peptide, and dried at 37 °C. 50 µl of anti-TPA:B1 IgG, diluted to 0.012 mg/ml, and of preimmune rabbit IgG, at the same concentration, were added to each well. Goat anti-rabbit horseradish peroxidase (GAR-HRP) (50 µl, 1:1000) was used as secondary antibody, and o-phenylendiamine (100 µl) as substrate. The enzymatic reaction was allowed to proceed for 10 min, then stopped by addition of 25 µl of 4 N $H_2SO_4$. The adsorbances of the wells were then read at 492 nm in a GDV 'Immunella'. Peptides B (HLA-DQ2 63–79) and G (HLA-DQ2 96–110) selected from Class II histocompatibility antigens, were used as negative controls.

*B* Binding of IgG isolated from commercial anti-TPA:B1 by affinity chromatography on Sepharose-peptide 4, as derived from ELISA. All conditions as in *A*. – – indicates controls with preimmune rabbit IgG at the same concentration.

five synthetic peptides, calculated according to Hopp and Woods[8], was −0.20; +0.16; +0.67; +0.33; +0.56 respectively. The commercial rabbit anti-TPA:B1 antibody (Byk Gulden, Milano) used for the identification of linear determinants in the BrCN:B fragment, was first purified from bovine serum albumin added as protecting agent by chromatography on a Bio Rad Affi Gel-Blue column in 0.02 M $K_2HPO_4$. The first peak eluted (IgG) corresponded to approx. 1/10 of the total protein amount loaded. This enriched antibody preparation with known protein concentration was then tested in an ELISA on the five synthetic fragments using preimmune rabbit IgG at the same dilutions as a control. The binding of this sample to the five peptides, as well as to 2 synthetic fragments selected from unrelated proteins used as controls, is shown in the figure. Anti-TPA antibodies appeared to react with peptides 4 and 5 only, corresponding to the C-terminal part of the BrCN:B fragment. An enrichment in anti-4 and anti-5 antibodies could be obtained by loading the commercial sample on a 0.5 × 5 cm column packed with an immunoadsorbent prepared by linking Sepharose AH to peptide 4 by aid of glutaraldehyde, and equilibrated with phosphate-saline buffer (PBS); 0.2 M Gly-HCl buffer pH 2.6 eluted a protein peak that was retested, under the same conditions, on the same set of synthetic peptides. The binding of the antibodies to the COOH-terminal fragment was more apparent in this case (fig.).

The antigenic sites of proteins are of two types, structurally: they may comprise residues that are on a continuous segment of the protein chain[9] or are far apart but, due to the folding of the polypeptide chain, come into close spatial proximity (discontinuous sites)[10]. Antibodies to an intact protein may be directed against both site structures[11]. Since commercial anti-TPA antibodies appear to bind large synthetic peptides closely resembling TPA BrCN:B fragment, and recognize an epitope centered around Arg 15[5] while they fail to react with short-sized synthetic peptides that include this residue, this finding strongly suggests that this antigenic determinant is conformational; however, we found that anti-TPA antibodies display an affinity for the C-terminal part of the fragment, where a continuous epitope therefore should be located.

The lack of binding of anti-TPA antibodies to synthetic fragments 1, 2 and 3 might have different alternative explanations: those linear sequences might display low immunogenicity in the intact TPA molecule, and elicit undetectable amounts of antibodies; alternatively, they might assume, in the native protein, a poorly flexible conformation, and elicit antibodies to this precise conformation but unreactive with random-coiled synthetic peptides. Finally, they might be buried or masked in the native molecule. This last possibility might be true for fragments 1–10 that display a negative H.I.[8], but not for the other two peptides that, because of their positive indices, (H.I. +0.16, +0.67, respectively), are probably located on the outside of the TPA molecule: peptides 2, 3, 4 and 5 might be used therefore for rabbit immunization and production of antipeptide antibodies directed to predetermined regions of TPA. However, peptides 2 and 3 share a more than 83% and a 72% homology, respectively, with human 50 K keratin, and it is predictable that antibodies to those peptides would extensively crossreact with these filamentous proteins, and therefore be of limited application. In contrast, peptide 4 (homology to keratin: 53%) and especially 5 (homology: 33%) might generate specific anti-TPA antibodies and be of considerable help in determining TPA amounts levels in sera of suspected cancer patients. Efforts are being made in our laboratory to raise an immune response against peptides 2, 3, 4 and 5, and detect whether the corresponding antipeptide antibodies can be used as specific anti-TPA reagents.

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1 Bjorklund, B., and Bjorklund, V., Int. Arch. Allergy *10* (1957) 153.
2 Bjorklund, B., Bjorklund, V., Wiklund, B., Lundstrom, R., Ekdahl, R. H., Hagbard, L., Kaiser, K., Eklund, G., and Luning, B., Immunological technique for detection of cancer, p. 133. Bonnier, Stockholm 1973.
3 Menendez-Botet, C. J., Oettgen, H. F., Pinsky, C. M., and Schwartz, M. K., Clin. Chem. *24*/6 (1978) 868.
4 Redelius, P., Luning, B., and Bjorklund, B., Acta chem. scand. B*34* (1980) 265.
5 Luning, B., and Nilsson, U., Acta chem. scand. *37* (1983) 731.
6 Merrifield, R. B., J. Am. chem. Soc. *86* (1964) 304.
7 Yajima, H., Ogawa, H., Watanobe, H., Fujii, N., Kurobe, M., and Miyamoto, S., Chem. Pharm. Bull. *23* (2) (1975) 371.
8 Hopp, T. P., and Woods, K. R., Proc. natl Acad. Sci. USA *78* (1981) 3824.
9 Atassi, M. Z., Immunochemistry *12* (1975) 423.
10 Atassi, M. Z., Immunochemistry *15* (1978) 909.
11 Berzofsky, J. A., Buckenmeyer, G. K., Hicks, G., Gurd, F. R. N., Feldmann, R. J., and Minna, J., J. biol. Chem. *257* (1982) 3189.

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## Distribution of tunichrome and vanadium in sea squirt blood cells sorted by flow cytometry

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*Summary.* Specialized blood cells of many tunicates accumulate high concentrations of vanadium and phenolic peptide pigments called tunichromes (TC). In order to determine whether V and TC reside in the same cells, *Ascidia nigra* and *Ascidia ceratodes* blood cell subpopulations were isolated by fluorescence-activated cell sorting (flow cytometry) and chemically analyzed. V was found in the spherical, green/grey signet ring cells, and to a lesser degree in the mulberry-shaped, yellow/green morula cells (MRs), whereas free TC was detected mainly in MRs.
*Key words.* Fluorescence activated cell sorting (FACS); tunicates; morula cells; signet ring cells; vanadium.