

EPITOPE ANALYSIS OF THE ALLERGEN OVALBUMIN (Gal d II) WITH MONOCLONAL ANTIBODIES AND PATIENTS' IgE

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Abstract—Ovalbumin (OVA) is a major allergen (Gal d II) of hen egg white and is often the cause of hypersensitivity reactions to food. Further knowledge of the antigenic and allergenic epitopes of allergens will provide better treatment of this disease. To analyse these epitopes we produced a panel of monoclonal antibodies (mAbs) against native OVA. The initial information about the epitopes was obtained with the binding patterns of these mAbs in IEF-immunoprints and western blots of OVA under reducing and non-reducing conditions. It was possible to demonstrate that the different conformations of OVA exhibit different epitopes, and that there are other epitopes which are shared by each conformation. Seven different, although sometimes overlapping epitopes, could be determined on native OVA; four different epitopes on denaturated non-reduced OVA by means of immunoblots of the intact molecule. The number of epitopes which could be differentiated by the mAbs was increased by the use of peptide blots after CNBr fragmentation of the molecule. IgE binding to different OVA conformations and to CNBr-fragments of OVA was also detectable and appears in the same regions as the reactivity of some mAbs. Western blots of OVA and CNBr-peptides demonstrate that some antigenic/allergenic binding sites seem at least partly to be continuous epitopes. The identification of the CNBr-fragments was performed by a microsequence analysis of blotted CNBr-fragments after a 2-dimensional electrophoresis. IgE was found to bind the two largest CNBr-fragments (residues 41-172 and 301-385), but not the fragment corresponding to residues 173-196. A number of monoclonal antibodies also reacted with the two large fragments, especially with fragment 301-385, and some bind also to shorter peptides, such as fragment 173-196, which were not reactive to patients' IgE. Most of the monoclonal antibodies and patients' IgE bind to the fragments 41-172 and 301-385 in 2D-PAGE blots suggesting that these fragments are involved in an immunogenic structure.

INTRODUCTION

Ovalbumin (OVA) is one of the major allergens of hen eggwhite (Langeland, 1982a, b, 1983; Hoffman, 1983; Holen and Elsayed, 1990), which is often the cause of hypersensitivity reactions to food (Metcalf, 1984; Jorde *et al.*, 1989). OVA is a very suitable model allergen for studying the relation between structure and function because the sequence of amino acids and post-translational modifications of the molecule are known (Nisbet *et al.*, 1981). The latter affect an *N*-terminal acetyl group, the position and kind of carbohydrates as well as the position of phosphoryl groups. The molecular structure of OVA based on X-ray crystallography has been reported by Stein *et al.* (1990) and the structure of plakalbumin, which is a proteolytic cleavage product of OVA (residue 1-358), was recently described by Wright *et al.* (1990).

Knowledge of the molecular basis of allergenic determinants may lead to an increased understanding of allergy and may contribute to better diagnosis and treatment of disease e.g. by blocking of the allergic

reaction with haptenic peptides or by inducing protective antibodies by other haptenic peptides of the molecule. A number of studies are available dealing with the antigenic and allergenic epitopes of OVA (Elsayed *et al.*, 1986, 1988, 1989, 1991; Johnssen and Elsayed, 1990). It was shown in RAST-inhibition tests that CNBr-cleavage products of OVA reveal allergic reactivity (Elsayed *et al.*, 1986). Synthetic peptides of OVA like the *N*-terminal decapeptide (Elsayed *et al.*, 1988) could inhibit the binding of IgE from patients' sera in RAST inhibition test, but failed to react *in vivo*, suggesting that this decapeptide encompasses an Ig-binding haptenic epitope. This is also the case with the peptide OVA 323-339 (Johnssen and Elsayed, 1990). Beyond that, it was shown that this peptide is a T-cell epitope of OVA (Buus *et al.*, 1986) in mice. Eight peptides in the region 11-122 of OVA were studied for their antigenicity and allergenicity and it was shown that some of these peptides are probably recognized by the antigenic binding sites of immunoglobulins (Elsayed *et al.*, 1991). These studies first and foremost gave information about continuous epitopes and although the use of peptides for studying antigenic/allergenic epitopes is under controversial discussion (Van Regenmortel, 1989; Jorde *et al.*, 1990), studies with natural or synthetic protein fragments have made it possible to locate antigenic and allergic regions on proteins (Trifileff *et al.*, 1991; Elsayed *et al.*, 1991).

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Monoclonal antibodies (mAbs) are of growing importance in allergen standardization and characterization, and are a suitable tool in the epitope analysis of allergens (Chapman *et al.*, 1984; Becker, 1987; Mourad *et al.*, 1988; Chapman, 1988; Petersen *et al.*, 1989; Marc-Series *et al.*, 1990; Lin *et al.*, 1990).

The aim of our study is to characterize antigenic and allergenic epitopes of OVA by means of mAbs by comparative binding studies in immunoblots obtained by different electrophoretic techniques. Most of the studies mentioned above (Marc-Series *et al.*, 1990; Lin *et al.*, 1990; Chapman *et al.*, 1984) used SDS-PAGE blots for identifying the allergenic components of extracts and mAb binding to these components. In our experience the IEF/immunoblotting technique is a very powerful, highly resolving technique for determining the immunoglobulin reactivities of patients' sera to various allergenic sources and testing the specificity of mAbs under conditions where the protein is not denatured (Becker, 1989). In this study, we used these techniques for initiating the characterization of antigenic binding sites on the intact molecule OVA with monoclonal antibodies. In order to localize the antigenic allergenic binding sites in more detail, the molecule was cleaved by CNBr and the binding studies were extended to blotted CNBr-fragments of OVA. The identification of CNBr-fragments was performed by microsequencing of blotted peptide spots which were separated by 2D electrophoresis. In order to determine that the monoclonal antibodies bind to allergenic relevant epitopes, we compared the binding patterns in immunoblots with those of IgE from patients allergic to hen egg-white.

MATERIALS AND METHODS

OVA (Fraction V, A 5503) was purchased from Sigma, Munich. The sera of patients allergic to hen egg-white were obtained from different sources: some are from patients of the Medical Clinic of Borstel, others were kindly provided by Prof. Dr U. Wahn (Bonn), P.D. Dr G. Schultze-Werninghaus (Frankfurt).

Monoclonal antibodies

Monoclonal antibodies against OVA were prepared according to Köhler and Milstein (1975) and Köhler (1980) using Balb/c mice and the myeloma cell line P3X63 Ag8U1. Growing clones were checked on their production by employing the Dot-test: 1 µl of a solution of 1 mg OVA/ml was applied to nitrocellulose strips (4 × 20 mm), dried and free binding sites were blocked with 0.05% Tween in Tris-buffered-saline (TBS: TBS-Tween: 0.1 M Tris, 0.1 M NaCl, 2.5 mM MgCl₂, 0.05% Tween-20, pH 7.4). 100 µl of culture supernatant was transferred from the culture plate (96 well plate) in microtiterplates and diluted 1:1 with TBS-Tween. The small strips were placed in the wells and incubated overnight. The following steps were the same as described under *Immunoblotting analysis* in this paper. The isotypes of the mAbs were examined using the Ouchterlony technique.

CNBr-cleavage of OVA

CNBr-cleavage of OVA was performed for 20 hr at room temp in 70% (v/v) formic acid according to Elsayed (Elsayed *et al.*, 1986) and Gross (1967). Samples of 5.55 µmol OVA and 9.4 mmol CNBr were used. The cleavage products were lyophilized and stored at -20°C.

Isoelectric focusing

Isoelectric focusing was performed on 0.9% agarose gel according to Haas *et al.* (1986) supplemented with 12% sorbitol (w/v). The ampholytes were 7.5% (v/v) Servalyt (3-10) and 2.5% (v/v) Servalyt 4-6 (Serva, Heidelberg). Focusing was performed on Ultraphor 2217 (LKB, Bromma) for 1,200 Vh at 4°C. OVA (10-20 µg) or 100-200 µg CNBr-cleavage products of OVA were applied. Marker protein test mix 9 was supplied from Serva, Heidelberg. Voltage was limited to 2,000 V, power to 35 W, current to 20 mA. Protein staining of IEF-gels was performed with Coomassie-brilliant blue and crocein scarlet according to Righetti and Drysdale (1974).

SDS-polyacrylamide gel electrophoresis

SDS-PAGE of OVA was performed according to Neville and Glossmann (1974) in a vertical slab gel, with a gradient of 7.5-20% acrylamide (7.5-20% T, 0.75% C) under non-reducing and reducing conditions. Gel was applied (15 µg OVA/cm). Reduction and alkylation was performed with dithioerythritol and vinylpyridine (Heukeshoven and Dernik, 1987).

SDS-PAGE for separation of CNBr-cleavage products of OVA was performed by a slight modification of Wülfang *et al.* (1988) and Kratzin *et al.* (1989). This SDS-PAGE-system uses $-\text{SO}_4^-$ as leading ion with Bis-Tris and Tris as counter ions in the stacking and separation gel. The trailing ion is Bicine with Bis-Tris as counter ion. Briefly: cathode buffer: 0.2 M Bicine (N,N-bis[2-hydroxyethyl]-glycine; Sigma, Munich), 0.1% SDS (w/v), pH 8.2. Anode buffer: 0.2 M Tris H₂SO₄ pH 8.1. Separation gel: 15% T and 5% C acrylamide, 0.4 M Tris H₂SO₄, 0.1% (w/v) SDS, 0.35 ml 87% glycerol/ml gel solution, 0.1% TEMED, 5 µl of a 10% APS-solution, pH 8.7. Stacking gel: 5% T and 5% C acrylamide, 0.4 M Bis-Tris/H₂SO₄, 0.1% SDS, 0.1% TEMED, 5 µl 10% APS-solution/ml gel, pH 6.7. Sample buffer: 179.5 mM Bis-Tris, 84.5 mM Bicine, 0.25% SDS, 1.25% mercaptoethanol, 10% saccharose, 0.02% bromophenol blue as tracking dye. Cleavage products 25-50 µg of OVA/cm were applied. We used a calibration kit for polypeptide mol. wt determination with a range of 2.5-17 kDa (Pharmacia, Sweden) as marker peptides. The gels were stained with silver nitrate according to Heukeshoven and Dernik (1986).

IEF-immunoblotting

Proteins from the gel were transferred to blotting membranes by the capillar blot technique according to Peltre *et al.* (1982). After IEF, a blotting membrane was

applied to the surface of the gel and the proteins were transferred by pressure (5 min, 1 kg; 5 min, 5 kg) by the covering with paper towels. Free binding sites were blocked with TBS-Tween. For blotting of OVA, nitrocellulose membranes (Schleicher and Schüll) with pore sizes of 0.45 μm were used. For peptide blotting these membranes were activated with CNBr according to Demeulemester *et al.* (1987), because peptide immobilization was detectable only on this kind of material.

Western blot

Western blot was performed by semi dry electroblotting according to Kyhse-Andersen (1984) with a Semi-Dry-Blottingsystem (Biometra Fast Blot System, Typ B 33, Biometra, Göttingen) with a current of 0.8 mA/cm² for 30 min. Free binding sites were again blocked with TBS-Tween pH 7.4.

Immunoblotting analysis

For the detection of monoclonal antibody binding 3 mm wide strips of the blotting membranes were incubated overnight with 2 ml of culture supernatant and 2 ml TBS-Tween. For each mAb there was evidence that the concn was in a range where all binding sites were saturated. After 3 washes in TBS-Tween pH 7.4 the strips were incubated with an alkaline phosphatase conjugated goat-anti-mouse IgG (specific for H and L chains; Jackson Immuno Research) diluted 1:5000 with TBS-Tween for 3 hr. MAb binding was made visible by the enzymatic reaction of alkaline phosphatase by incubating the strips with a substrate-solution of nitro blue tetrazolium (NBT) 0.033% and 5-bromo-4-chloro-indolylphosphate disodium (BCIP) 0.5% in TBS without Tween, pH 9.5.

The binding of IgE from patients sera was performed by incubating the strips with human sera diluted 1:20 with TBS-Tween, pH 7.4 overnight. After three washes with TBS-tween, IgE binding was made visible by incubation with a J¹²⁵-labelled rabbit-anti-human-IgE (Phadebas-RAST, Pharmacia) approximately 1×10^6 cpm for 15 strips for 4 hr and subsequent autoradiography on a Kodak X Omat film for 3, 7 and 14 days.

Protein staining on the blotting membranes was performed with a solution of 0.1% India Ink (Pelikan Fount India) in TBS-Tween according to Hancock and Tsang (1983).

2-Dimensional electrophoresis (2D-SDS-PAGE)

The 2D-electrophoresis was performed with immobilized pH-gradients according to Görg *et al.* (1988) and Kinzkofer-Peresch *et al.* (1988). The gel size for both dimensions was 22 cm $\times$ 24.5 cm $\times$ 0.5 mm. In the first dimension an IEF in gels casted on GelBond-PAG (LKB, Sweden) with immobilized pH-gradient ranging from 3.6–9.3 pI and an acrylamide concn of 4% T and 4% C was performed. After polymerization, the gel was dried by microwaves and stored at -20°C . 4 mm wide gel-strips were incubated in a rehydration buffer (8 M

urea, 0.5% (v/v) Nonidet-P 40, 10 mM dithiothreitol, 0.4% (v/v) Servalyt 3–10, 0.1% (v/v) Servalyt 4–6) overnight. The IEF was performed on a Desaphor HF electrophoresis unit (Desaga, Heidelberg, Germany). For sample entry voltage was limited to 300 V for 1 hr, the separation was performed at 3000 V, 2 mA and 5 W for 7 hr. CNBr-cleavage products (200–400 μg) were applied. The strips were stored at -80°C until they were used in the second dimension. In the second dimension an SDS-PAGE was performed on GelBond-PAG stabilized gels with acrylamide concns from 10–15% T and 4% C in the separation gel and 5% T, 4% in the stacking gel. The gel strips were incubated 2 $\times$ 15 min in equilibration buffer (0.05 M Tris, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, traces of Bromophenol Blue, 1% (w/v) dithiothreitol. Iodoacetamide 260 mM were added to the buffer in the second incubation step. The equilibrated gel strips were transferred gel side down on the SDS-PAGE gel. The electrophoresis was performed on a 2117 Multiphor II electrophoresis unit (LKB, Sweden) with maximum settings of 200 V, 30 A and 30 W until the probes left the stacking gel, then voltage was limited to 800 V. For electrophoretic transfer of the peptide pattern on blotting membranes the same semi-dry-blotting system was used as described above. For immunologic detections PVDF-membranes (Immobilon-P) were used.

Microsequence analysis

Amino acid sequence analysis was performed on an Applied Biosystems (California) 473A pulse liquid sequencer. 200–400 μg of CNBr-cleavage products were separated by 2D electrophoresis and electroblotted on ProblottTM-membranes (Applied Biosystems, California) using CAPS-blotting buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, 10% (v/v) methanol, pH 11) according to Matsudeira *et al.* (1987). The spots were stained with 0.1% (w/v) Coomassie in 40% (v/v) methanol and destaining of the background was performed with 50% (v/v) methanol. Stained spots were cut out of the dried blotting membrane and arranged in the cartridge block of the sequencer. Because the amino acid sequence of OVA is known, only 5–10 amino acids from the N-terminal end of the peptides were analyzed.

RESULTS

OVA and CNBr-cleavage of OVA

The staining of the gel after SDS-PAGE under non-reducing conditions showed four bands with an apparent mol. wt of 40, 45, 63 and 72 kDa, with the strongest at 45 kDa, whereas under reducing conditions only one band at 45 kDa could be obtained. IEF of OVA yielded about 3 bands after the gel staining. Up to 10 bands were detectable after the transfer of the protein onto nitrocellulose membranes and protein staining with India ink. Treatment of OVA and CNBr led to a complete cleavage into smaller peptides. About 16 bands with mol. wts between 1000 and 16,500 Da were detectable.

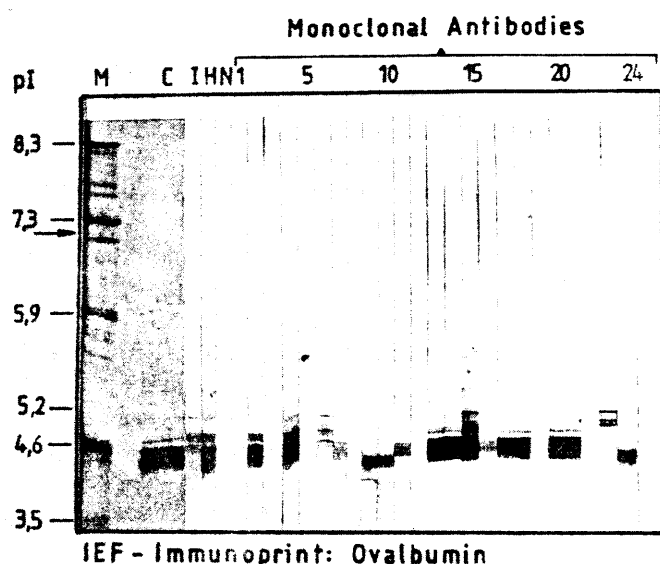


Fig. 1. Binding of mAbs on immunoprint of OVA on nitrocellulose membrane, poresize $0.45\ \mu\text{m}$. pI = isoelectric point. M = marker, C = protein staining with Coomassie, H = hyperimmune serum, N = negative control, 1–24 = reactivities of 24 hybridoma culture supernatants.

Monoclonal antibodies

Twenty-four hybridomas producing monoclonal antibodies against OVA were selected. The isotype was confirmed by the Ouchterlony technique. MAb nos 1, 2, 3, 5, 7, 8 and 19 belong to the IgM isotype, the others to the IgG1 isotype.

Reactivities to the intact molecule OVA were tested in IEF-immunoprints and western blots under reducing and non-reducing conditions. Figure 1 shows the binding patterns in IEF-Immunoprints of OVA. Altogether 7 different reaction patterns could be differentiated: three mAbs (nos 2, 4, 15) bound the whole spectrum of bands as did hyperimmune serum in a range of pI 4.2–4.9.

Twelve of the mAbs (nos 1, 3, 7, 8, 14, 17, 18, 19, 20, 21, 22) showed reactivities to fewer bands around pI 4.4–4.7. Three mAbs (nos 5, 11, 16) showed a more limited binding pattern at pI 4.6. Two mAbs (nos 6, 23) bound bands in a more basic region from pI 4.7–4.9; these two mAbs differ from each other in their binding patterns. Four mAbs (nos 9, 10, 12, 24) bound in a more acidic region around pI 4–4.4. When comparing the binding pattern in western blots of OVA under non-reducing conditions (Fig. 2) it can be shown that special binding patterns in IEF-immunoprint correspond to distinct binding patterns in the western blots under non-reducing conditions. The four mAbs (nos 9, 10, 12, 24) which bound in the acidic region of OVA after separation by IEF showed reactivities exclusively at an apparent mol. wt of 63 kDa; the two mAbs which bound in the basic part of OVA in immunoprints showed reactivities only at 55 kDa. The mAbs which recognized the whole spectrum of bands of IEF-separated OVA have the same qualities in western blots under non-reducing conditions. The other mAbs revealed the main reactivity to the strongest band at 45 kDa. The reducing conditions led to one band at 45 kDa and all mAbs bound to that band with a more or less great intensity.

In western blots of the peptides, the binding of mAbs could be detected solely on Immobilon membrane (PVDF-membrane, Millipore) (Fig. 3). Seven bands in a range of 3.8–16.5 kDa could be obtained by immunological detection with hyperimmune serum and mAbs. Some of the mAbs (nos 1, 2, 3, 7, 19, 21, 22) showed strong reactivities, but always with more than one band. They differed from each other in the intensity of binding to the separate bands. After the IEF, about 19 bands of the CNBr-cleavage products in a range of pI 4.2–8.3 were detectable by protein staining of the gel with Coomassie. Only the CNBr-activated membranes are suitable for the transfer of the peptides of capillary blotting. All peptides were transferred to the membrane.

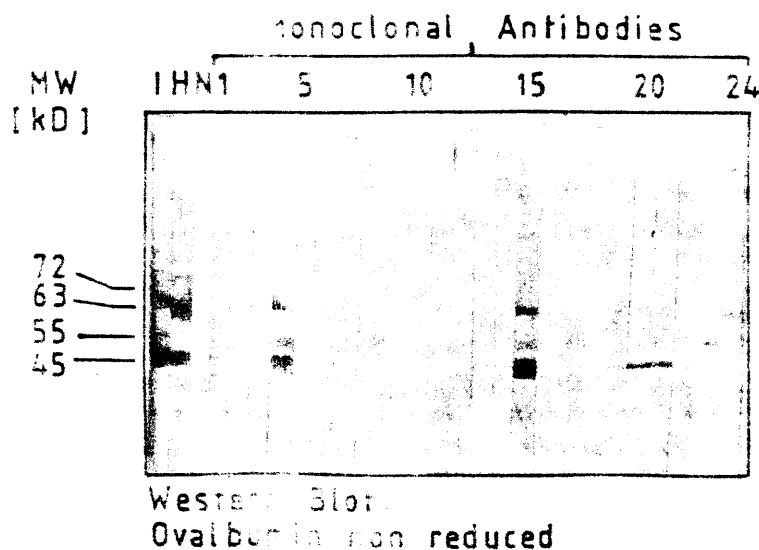


Fig. 2. Binding of mAbs on western blot of OVA under non-reducing conditions on nitrocellulose membrane, poresize $0.45\ \mu\text{m}$. MW = molecular weight, I = protein staining with India ink, H = hyperimmune serum, 1–24 = reactivities of 24 hybridoma culture supernatants.

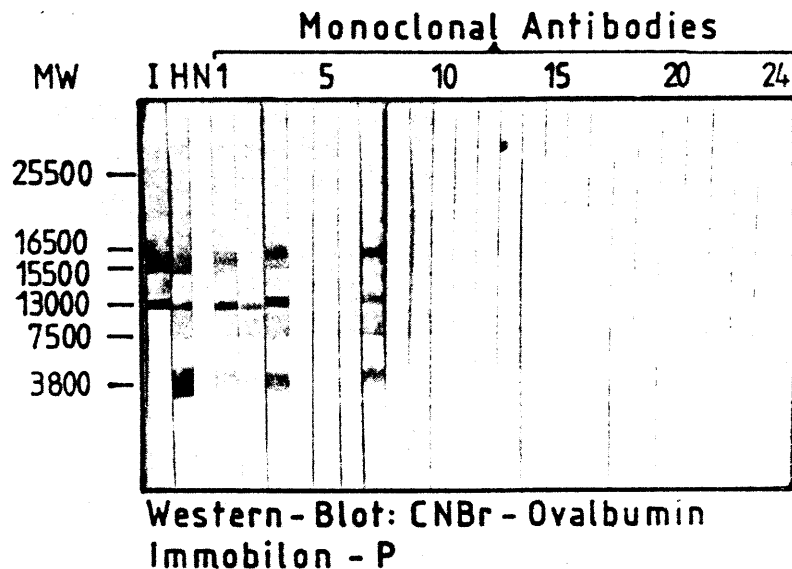


Fig. 3. Binding of mAbs on Western blot of CNBr-cleavage products of OVA under reducing conditions on PVDF-membrane (Immobilon). Mol. wt = molecular weight, I = protein staining with India ink, H = hyperimmune serum, 1-24 = reactivities of 24 hybridoma culture supernatants.

as evidenced when comparing the protein staining of the gel with membrane staining by India ink or immunological detection with hyperimmune serum (Fig. 4). Four mAbs (nos 9, 10, 12, 24) showed strong reactivities to the peptides separated by IEF, additionally there were three other mAbs (nos 11, 13, 19) with a much weaker reactivity. The four first mentioned mAbs were identical to those that bind in a more acidic region in the IEF-immunoprints of the intact molecule. The use of peptide blots was necessary to demonstrate that each of these mAbs recognized different but sometimes overlapping epitopes. The mAbs which revealed strong reactivities in the western blots of the peptides, showed

no reactivities in the IEF-immunoprints of the peptides. Vice versa, the mAbs which possessed strong reactivities in the IEF-immunoprints of peptides did not recognize the peptides under the conditions of SDS-PAGE. The results of these comparative binding studies are listed in Table 1.

IgE from patients' sera

In order to demonstrate that the binding of IgE from patients' sera occurs in the same regions as the mAbs, the reactivities of some individual sera from patients allergic to hen egg-white were tested together with some selected mAbs on identical blots. IgE-binding to the different conformations of OVA as well as to the cleavage products could be demonstrated by autoradiography both in western blots and immunoprints. Those sera showing IgE-reactivities in immunoblots of the intact OVA always exhibited reactivities with the cleavage products of OVA. The major binding of IgE with fragments occurred with three bands in a mol. wt range from 10 to 17 kDa. Another fragment with a MW of about 7-8 kDa exhibited IgE-binding too, but in a much weaker fashion. In the immunoprint of OVA-cleavage products the binding of IgE was detectable in the same regions where the four mAbs (nos 9, 10, 12, 24) showed strong reactivities. Especially the binding pattern of mAb-no. 9 was very similar to that of patients' IgE.

2-Dimensional electrophoresis and microsequencing

Figure 5 shows the pattern of CNBr cleavage-products after separation by 2D-SDS-PAGE and silver staining of the gel. The numbers indicate all spots which were submitted to microsequence analysis after transfer onto Problott-membrane and Coomassie staining. After the silver staining of the gel, more spots are detectable than by Coomassie staining of blotted fragments. Figure 6 shows the binding of patients' IgE to these fragments and Fig. 7, as an example, the binding of mAb-no. 2. The

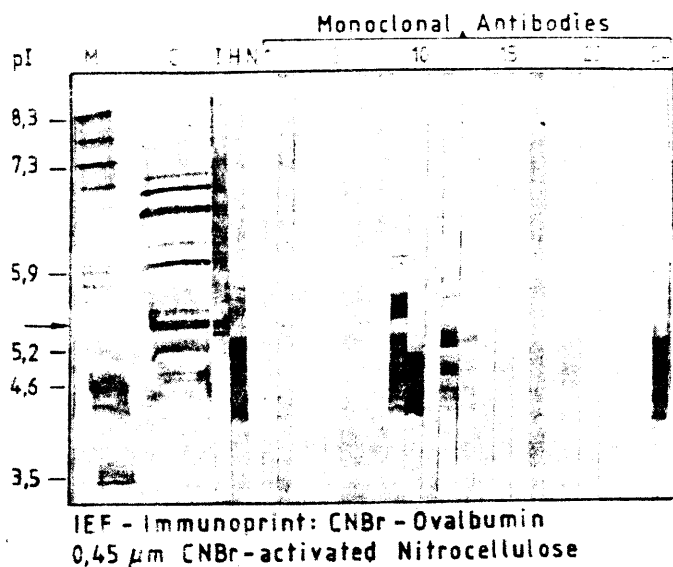


Fig. 4. Binding of mAbs on Immunoprint of CNBr-cleavage products of OVA on CNBr-activated nitrocellulose membrane, pore-size 0.45 µm. pI-isoelectric point, M = marker, C = protein staining with Coomassie, H = hyperimmune serum, N = negative control, 1-24 = reactivities of 24 hybridoma culture supernatants.

Table 1. Reactivities of monoclonal antibodies and patients' IgE in immunoblots.

mAb No.	1	2	3	4	5	6	7	8	9	10 ₂	11	12	13	14	15	16	17	18	19	20	21	22	23	24
IEF: OVA	C ₁	H	C ₁	H	C ₂	B	C ₁	C ₁	A	A	C ₂	A	C ₁	C ₁	H	C ₂	C ₁	C ₁	C ₁	C ₁	C ₁	C ₁	B	A
SDS-PAGE: OVA	C		C	H	C	B	C	C	A	A	C	A	C	C	H	C	C	C	C	C	C	C	B	A
IEF: CNBr-OVA	-	-	-	-	-	-	-	-	++	++	+	++	+	-	-	-	-	-	+	-	-	-	-	++
SDS-PAGE: CNBr-OVA	a	++	++		-	+	++	c	-	-		-	-	(+)	(+)	-	(+)	-	++	-	++	a	-	
Ig-class	M	M	M	G1	M	?	M	M	G1	G1	G1	G1	G1	G1	G1	G1	G1	G1	M	G1	G1	G1	G1	G1
2D-PAGE: Fragment 41 - 172	+	+	-		-	+	+	+	+	-	-	+	+	+	+	+	+	-	-	+	+	-	+	-
173 - 196	+	+	-	+	-	-	+	+	+	-	+	+	-	-	+	+	-	-	+	+	+	-	+	-
301 - 385					+	-	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	-
41 196 ?	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+

IEF: OVA: IEF-immunoprint of OVA; A, H = reactivities of mouse hyperimmune serum at 10 bands in a range from pI 4.2-4.9; C₁ = reactivities from pI 4.4-4.7; C₂ = reactivities at pI 4.6; B = reactivities from pI 4.7-4.9; A = reactivities from pI 4.0-4.9.

SDS-PAGE: OVA: Western blots of OVA under non-reducing conditions; H = reactivities like hyperimmune serum at bands with an apparent mol. wt. of 45,55,63 and 72 kDa; A = reactivity at mol. wt. 67 kDa; B = reactivity at mol. wt. 55 kDa; C = strong reactivity at 45 kDa; weaker reactivity at 72 kDa. Under reducing conditions only one single band at 45 kDa appears and the mAb find in a more or less intensity to that single band.

IEF: CNBr-OVA: IEF-immunoprint of CNBr-cleavage products of OVA; [++]= strong reactivity with bands in a range of pI 4.2-5.9; [+] = weak reactivity with bands in a range of pI 4.4-5.2; [-] = no reactivity.

SDS-PAGE: CNBr-OVA: SDS-PAGE of CNBr-cleavage products of OVA; [++]= strong reactivity; [+] = medium reactivity; [-] = weak reactivity; [-] = no reactivity. Four different reaction patterns designed as a, b, c, d.

2D-PAGE: CNBr-OVA: Identification of CNBr-cleavage products by microsequence analysis of blotted peptide spots; mAb binding to the respective CNBr-fragment. [++]= the mAb showed a reactivity; [-] = no reactivity. "A" is a spot which could not be identified by microsequence analysis.

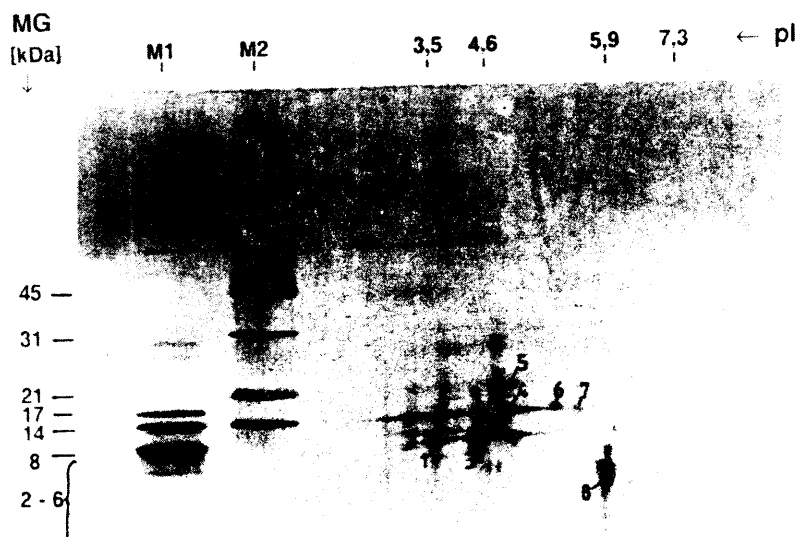


Fig. 5. 2D-SDS-PAGE of CNBr-cleavage products. Silverstaining of the gel. The numbers indicate spots which were applied for microsequence analysis.

results of the microsequence analysis of the marked spots are demonstrated in Table 2. Several peptides with different pI possess identical MW and *N*-terminal amino acid sequences. Five spots of CNBr-cleavage product consisting of amino acids 41–172 (mol. wt 16,000 Da) and two spots of CNBr-cleavage product amino acids 301–385 (mol. wt 10,300 Da) were identified and showed strong binding with patients' IgE. Amino acid sequence analysis of the whole peptide of spot 8 confirmed that it represents a fragment of complete CNBr-cleavage. It was identified as fragment 173–196 with a MW of 3064 Da. This fragment is immunoreactive with most of the mAbs (19 of 24 mAbs) but failed to show binding with patients' IgE. Other fragments in the low MW range from 2 to 6 kDa and pI 3 to 4 showed IgE binding, but these spots were not detectable by Coomassie staining on Problott-membrane and could therefore not be submitted to microsequence analysis. The reactivities of

mAb binding to the CNBr-fragments is also listed in Table 1. It is obvious that most of the mAbs show reactivities to more than one fragment, twelve mAbs bind to each of the three identified fragments (41–172, 173–196, 301–385). Some mAbs (nos 9, 20, 24) react with a broad spot at pI 3–3.5 and 30 kDa (signed A in Tables 1 and 2). Protein staining and patients' IgE failed to visualize this product.

DISCUSSION

The aim of our study is the determination of antigenic and allergenic epitopes of OVA. For this purpose we have used monoclonal antibodies and different electrophoretic techniques with subsequent immunoblotting. The mAbs were employed to characterize the epitopes in immunoblots of OVA under native and denaturing conditions. In order to encompass the immunogenic

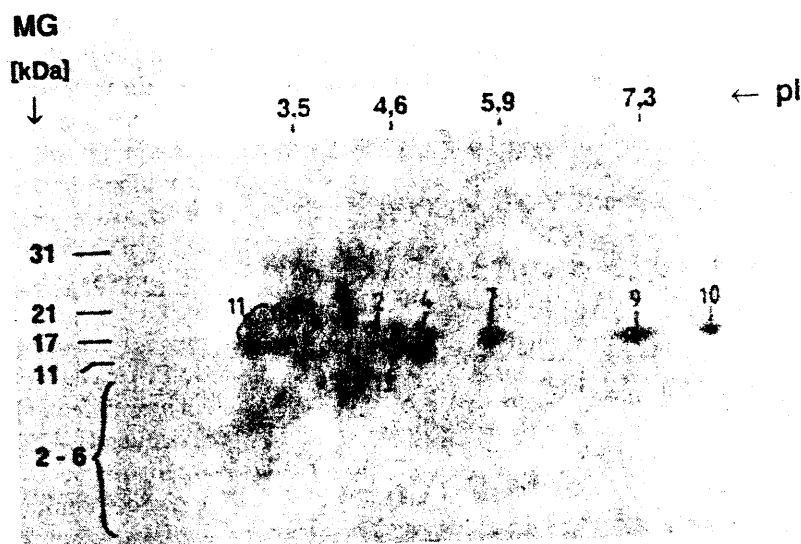


Fig. 6. 2D-SDS-PAGE of CNBr-cleavage products. IgE reactivity of a pool from sera of four allergic patients detected by autoradiographie on PVDF-membrane. The numbers indicate spots which were applied for microsequence analysis.

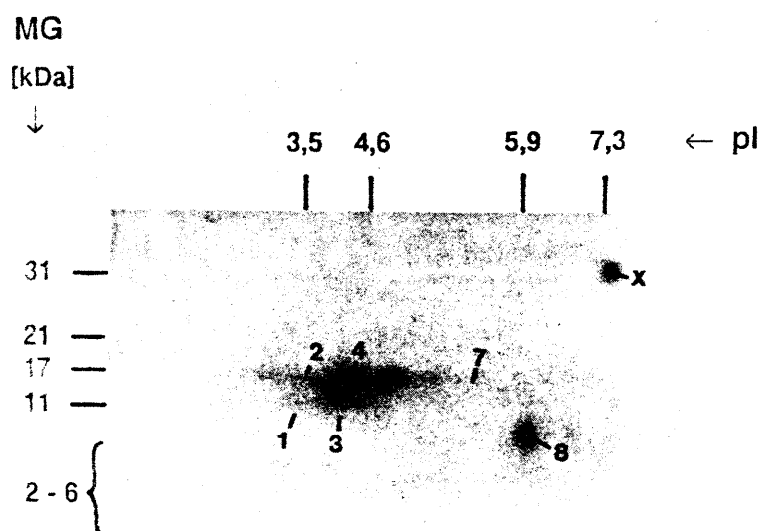


Fig. 7. 2D-SDS-PAGE of CNBr-cleavage products. Reactivity of mAb no. 2 detected by autoradiographie on PVDF-membrane. The numbers indicate spots which were applied for microsequence analysis, x = artifact.

parts of OVA, we extended the characterization with blots of CNBr-cleavage products of OVA. The identification of the CNBr-cleavage products was performed by a *N*-terminal sequence analysis of blotted fragments after separation by 2-D electrophoresis.

Separation of OVA by IEF yielded up to 10 bands after staining the nitrocellulose membrane with India Ink, whereby the bands demonstrated a micro-heterogeneity of the protein. With the binding patterns of the mAbs, seven different, although sometimes overlapping epitopes could be detected on native OVA.

SDS-PAGE of OVA under non-reducing conditions leads to 4 bands which is due to the variable location of an intramolecular disulphide bond (Fothergill and Fothergill, 1970). Under reducing conditions only one band of OVA is obtained. Thus different conformations of OVA exist: the disulphide interchain may be responsible for the conversion of newly synthesized "R-ovalbumin" to the more stable "S-ovalbumin" in storage (Smith, 1964; Nisbet *et al.*, 1981). All mAbs still recognize OVA under the denaturing conditions of SDS-PAGE. Certain binding patterns of non reduced OVA in SDS-PAGE (e.g. 55 kDa) correspond to certain binding patterns in IEF-immunoprint (e.g. basic region of OVA from pI 4.7–4.9) demonstrating that some epitopes are associated only with one conformation whereas others are common to each conformation. Four different epitopes are detectable on denatured OVA under non reducing conditions. All mAbs bind to OVA under reducing denaturing conditions with a more or less great intensity suggesting that the epitopes of OVA are at least partly continuous epitopes. Each conformation exhibits IgE-binding epitopes both on the native or denatured allergen.

After treatment with CNBr 17 cleavage products of OVA are theoretically expected from which 11 possess a mol. wt higher than 800 Da and should therefore be accessible by the SDS-PAGE for peptides applied in this study (Wiltfang *et al.*, 1983). The results demonstrated

that after separation in IEF and SDS-PAGE a number of cleavage products larger than expected was obtained. Methodological considerations concerning the electrophoretic behaviour of small peptides, reaction conditions chosen for the protein cleavage and a micro-heterogeneity of the protein concerning the grade of glycosylation or an amino acid exchange may be responsible for these observations (Kratzin *et al.*, 1989; Nisbet *et al.*, 1981; Honda *et al.*, 1990).

Four mAbs (nos 9, 10, 12, 24) show strong reactivities with native CNBr-fragments of OVA as detected in immunoprint. Therefore these cleavage products seem to be associated with one of the conformations of OVA (63 kDa in SDS-PAGE, pI 4–4.4). The mAbs nos 9, 10, 12 and 24 do not show any reactivity to the cleavage products under the conditions of SDS-PAGE. On the other hand, some mAbs (nos 1, 2, 3, 7, 19, 21, 22) reveal reactivities to cleavage products in western blots but they are not able to respond to those in IEF-immunoprints. These epitopes seem to be associated with the denatured structure of OVA and it might be concluded that part of their recognition sites are sequential epitopes. With the peptide blots we were able to detect a greater number of differences in some of the antigenic determinants of OVA than with blots of the intact molecule. IgE reactivity to CNBr-cleavage products in western blots and immunoprints reveals that mAb binding seems to be associated with the same or closely related structures of OVA.

High resolution 2D electrophoresis allowed the identification of CNBr-cleavage products by *N*-terminal sequence analysis. Three different fragments corresponding to amino acid residues 41–172, 173–196 and 301–385 were identified. Some fragments in the lower mol. wt range of 2–6 kDa possess immunologic reactivity both with human IgE and mAbs, but these spots could not be visualized by Coomassie staining and thus a sequence analysis and identification was not possible. It could be demonstrated that several fragments with identical

Table 2. Reactivities of monoclonal antibodies and patients' IgE in immunoblots

Spot	N terminal sequence of CNBr cleavage products										Identification CNBr-fragment	MG [kDa]	pI	Patients' IgE
	Gly	Ile	Thr	Asp	Val									
1				Asp	Val						310 - 385	10.5	3.5	+
2	Ala Gly	Ser Ile Tyr	Leu	Gly Asp	Ala						Mixture 41 - 172 & 310 - 385	16.5	3.8	+
3	Gly	Ile	Thr	Asp	Val		Phe				310 - 385	10.5	4.3	+
4	Val	Tyr	Leu	Gly	Ala Asn		Ala		Phe		41 - 172	16.5	4.6	+
5	Val Ala	Leu?	Val?	Asn	Ala		Ala				173 - ?	20.0	4.6	-
6	Val	Tyr	Leu	Gly	Ala						41 - 172	16.5	5.0	-
7	Val	Tyr	Leu	Gly	Ala						41 - 172	16.5	5.5	+
8	Val	Leu	Val	Asn	Ala		Ile		Phe	Arg	173 - 196	3.0	5.8	-
9	Val	Tyr	Leu	Gly	Ala						41 - 172	16.5	3-3.5	+
10	Glu	Tyr	Leu	Gly	Ala		Lys		Asp		41 - 172	16.5	7.0	+
11	Val	Tyr	Leu Val	Gly Asn	Ala		Ile		Phe		41 - 196 ?	20.0	8.3	+
A											?	30.0	3-3.5	-

N terminal sequence detected from Coomassie-stained blotted peptide spots. Five-to-ten amino acids from the N-terminal end of the peptide spots were determined. OVA consists of 385 amino acid residues. Column of patients' IgE: | + | = reactivity with patients' IgE, | - | = no reactivity.

N-terminal sequence and mol. wt possess spots at different pI which might be due to chemical modifications during the cleavage procedure or microheterogeneity of the respective fragment, e.g. substitution of one amino acid residue by another one. Such an exchange is obvious in fragment 41–172 (spot 10 in Fig. 6) where Val at position 41 is substituted by Glu.

In contrast to the immunoblots of CNBr-cleaved OVA obtained from 1D-SDS-PAGE most of the mAbs showed reactivities with fragments in 2D-biots although this reactivity appears to be much weaker than that of patients' IgE. This may be interpreted in the following way: the peptides on 1D-strips were denatured by urea but not by heating. The urea may diffuse during the blotting and/or incubation procedures, allowing a re-folding of the larger peptides into their native structure. These structures could be recognized by most of the monoclonal antibodies demonstrating the requirement of a special conformation. This is also indicated by the observation that some mAbs show reactivities with the three different cleavage products which were identified by microsequence analysis, suggesting that each of these fragments is involved in one antigenic site of OVA or has at least cross reactive properties. This observation indicates again that most of the mAbs recognize conformational epitopes. The peptide in spot A is recognized only by the mAbs nos 9, 20 and 24. It seems to be immunogenic in the mouse but no human IgE reactivity was observed. Unfortunately it was not possible to identify this peptide by *N*-terminal sequence analysis because any protein staining failed. Spot 11 in Fig. 6 is a structure which is recognized by the patients' IgE but there was no respective binding of any mAb. Sequence analysis revealed that this fragment may be a product of incomplete cleavage or reassembling between CNBr-fragment 41–172 and 173–196.

The question arises, why only few mAbs recognize the cleavage products under the native conditions of IEF-immunoprints. A possible explanation may be the use of CNBr-activated nitrocellulose membranes because other membrane materials like untreated nitrocellulose or PVDF failed to immobilize peptides by the method of capillar blotting. The immobilization of peptides/proteins on CNBr-activated nitrocellulose is due to covalent binding which might result in a deformation of smaller protein fragments and thus hamper the recognition by most of the mAbs. In contrast, the patients' IgE showed reactivities on IEF-immunoprints of cleavage products which suggests that human IgE binding is preferentially associated with sequential protein structures. A further reason for this observation may be that only the polyclonal patients' IgE is able to give a strong signal in immunoprints of cleavage products whereas most mAbs do not.

The comparison of the immunoreactive fragments with information about the crystal structure of OVA (Stein *et al.*, 1990) and plakalbumin (Wright *et al.*, 1990) reveals that the reactive center loop of OVA (residue 338–357) as a member of the serpin-family is located on the fragment 301–385. This is a flexible α -helix

protruding from the molecule. Most of the mAbs showed reactivities with the respective fragment indicating that this structure might be very immunogenic. CNBr-cleavage product residue 41–172 contains a number of helices (helices hB, hC, hC2, hC3, hD, hE, hF) in its secondary structure which are located at the surface of plakalbumin (Wright *et al.*, 1990) and thus may contribute to the immunogenicity of this fragment. The observation that mAbs and patients' IgE showed binding to the fragments 41–172 and 301–385 indicates that these fragments contain structures that are very immunogenic both in mice and in man.

The fragmentation of OVA with endoproteinases or chemicals of another cleavage specificity than CNBr and binding studies with synthetic peptides of OVA should allow further identification of the antigenic and allergenic binding sites of OVA.

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