

Chicken antibodies to a recombinant fragment of the equine immunoglobulin epsilon heavy-chain recognising native horse IgE

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Abstract

An equine immunoglobulin E (IgE) heavy-chain cDNA fragment (CH3–CH4, nucleotides 1132 to 1592) was cloned, expressed in *Escherichia coli* as a fusion protein with a [His]₆-tag and purified over a Ni–NTA column. The recombinant protein was used to immunise hens. Testing of the raised egg yolk immunoglobulin G (IgG) in Western-blot and ELISA revealed high titres against the recombinant equine IgE fragment (reqIgEf). The reqIgEf-specific IgG was successfully affinity-purified on an unconventional affinity matrix: the [His]₆-tagged recombinant IgE fragment was bound to Ni–NTA agarose and used to adsorb specific immunoglobulins. In Western-blot of ammonium sulphate precipitated horse serum and bronchoalveolar lavage fluid, separated by

Abbreviations: Ab, antibody; AP, alkaline phosphatase; BALF, bronchoalveolar fluid; BCIP, 5-bromo-4-chloro-indolyl-phosphate; COPD, chronic obstructive pulmonary disease; DHFRS, dihydrofolate reductase; *E. coli*, *Escherichia coli*; ELISA, enzyme-linked immunosorbent assay; ESA, equine serum albumin; IgE, immunoglobulin E; IgG, immunoglobulin G; IPTG, isopropyl-β-D-thiogalactopyranoside; kDa, kilodalton; LB, Luria Bertani; NBT, 4-nitro blue tetrazolium chloride; Ni–NTA, nickel–nitrilotriacetic acid; OD, optical density; PCR, polymerase chain reaction; PNPP, *p*-nitrophenyl-phosphate; RBS, ribosomal binding site; reqIgEf, recombinant equine IgE fragment; RCA, reverse cutaneous anaphylaxis; RT, room temperature; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; T, Tween-20; TBS, tris-buffered saline; 2 × YT, 2 × concentrate of yeast extract tryptone broth

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SDS-PAGE under denaturing–reducing conditions, the raised antibodies reacted with a protein of approximately 80 kDa. A reaction of the reqIgEf-specific IgG was seen with a 190–200 kDa band when the same horse serum or bronchoalveolar fluid (BALF) was separated under non-reducing conditions. These reactions could be inhibited by preincubation of the immune IgG with reqIgEf, while preincubation with horse IgG did not inhibit the reaction. Antibody-affinity chromatography of horse serum with the reqIgEf-specific chicken IgG resulted in an enrichment of the 80 kDa protein in denaturing Western-blot. Determination of the amino acid composition of this protein and comparison with the equine IgE heavy-chain sequence strongly indicates that the 80 kDa band corresponds to the heavy chain of the horse IgE. The reqIgEf-specific chicken IgG was further characterised in an ELISA for the detection of allergen-specific horse IgE. It was demonstrated that heating IgE positive horse sera at 54°C for 10 min drastically diminished the recognition by the reqIgEf-specific chicken IgG. The reaction is inhibitable by preincubation with reqIgEf in a concentration dependent manner. In addition, preincubation with horse IgG, a nonrelevant [His]₆-tagged protein or 2% equine colostrum had no influence on the reqIgEf-specific chicken IgG binding characteristic. This antibody recognising horse IgE will be useful for further studies on the pathogenesis of equine allergic diseases. © 1997 Elsevier Science B.V.

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1. Introduction

Allergic diseases involving Type I hypersensitivity reactions, at least at certain stages of the disease, are not rare in the horse. Inhalation allergens (*Aspergillus fumigatus*, *Alternaria*, etc.) or insect bite allergens (*Culicoides* spp., etc.) seem to be the etiological agents for chronic obstructive pulmonary disease and summer dermatitis, respectively. Studies on the significance of immunoglobulin E (IgE) in the pathogenesis of these diseases have been hindered by the lack of horse specific reagents. However, an involvement of IgE-mediated, immediate Type I reactions in the above mentioned diseases was demonstrated indirectly using skin tests (Evans et al., 1992), in vitro degranulation of basophils stimulated with specific allergens (Gerber et al., 1982; Dirschel et al., 1993) and by the study of the bronchoalveolar fluid (BALF) of chronic obstructive pulmonary disease (COPD)-affected horses (Halliwell et al., 1993).

IgE molecules are responsible for the release of inflammatory mediators in immediate type (Type I) hypersensitivity reactions in humans and in other species (Ishizaka and Ishizaka, 1975). Thus, the prerequisite for investigations on the pathogenesis of Type I allergic conditions and for their in vitro diagnosis is the availability of specific antibodies raised against IgE as well as purified IgE as immunoreagents. The extremely low concentration of IgE in serum makes the isolation of pure IgE difficult. The discovery of human myeloma IgE (Johansson and Bennich, 1967), immunocytoma rat IgE (Bazin et al., 1974) and the construction of a mouse IgE hybridoma (Böttcher and Hämmerling, 1978) allowed the production of highly specific polyclonal and monoclonal antibodies in these species. In the horse, however, no IgE gammopathy has been discovered as yet.

Equine IgE-specific polyclonal antisera were prepared by Suter and Fey (1981) and by Halliwell and Hines (1985). In both investigations horse IgE was isolated from horse

serum by chromatography and used to immunise rabbits. The antisera produced were absorbed with other equine immunoglobulin classes. These studies strongly suggest the existence of low levels of IgE in horse serum. These anti-IgE reagents are not available commercially.

cDNA sequences of the equine epsilon gene have been published recently (Marti et al., 1995; Navarro et al., 1995), thus laying the foundations for the production of horse IgE constant region by recombinant DNA technology.

The aim of this study was to express an equine IgE heavy-chain fragment in *Escherichia coli* and to use it to produce antibodies recognising both the recombinant IgE molecule and native horse IgE. For this purpose, a goat, two rabbits and six chickens were immunised with the recombinant protein and the antisera raised were characterised. This paper describes the experiments carried out with egg yolk immunoglobulin G (IgG), because only the antibodies produced in hens recognised native horse IgE.

2. Material and methods

2.1. Production of a recombinant equine IgE heavy-chain fragment

Primers were designed in order to amplify a part of the constant region (CH3–CH4) of the equine IgE corresponding to nucleotides 1132 to 1592 of the equine IgE sequence published by Navarro et al. (1995). A *Bam*HI and a *Hind*III restriction site were incorporated into the forward and reverse primer, respectively, for unidirectional cloning. The DNA template used was the previously described and sequenced cDNA clone in plasmid pT7Blue(R)T (Marti et al., 1995). The same PCR conditions as described earlier (Marti et al., 1995) were used. The 465-bp PCR product was purified with GenElute™ Agarose Spin Columns (SUPELCO, Bellefonte, PA, USA), digested with *Bam*HI and *Hind*III and ligated into the *Bam*HI/*Hind*III cleaved vector PDS781/RBSII, [His]₆-DHFRS using standard protocols (Sambrook et al., 1989). The plasmids were introduced into *E. coli* M15 cells by electroporation using a Gene Pulser® (Bio-Rad Laboratories, Glattbrugg, Switzerland). Transformants were selected on LB-plates containing 100 µg ampicillin and 25 µg kanamycin per ml. A clone containing the correct insert as shown by sequence determination was used for further work.

E. coli cells containing the vector PDS781/RBSII, [HIS]₆ (Stüber et al., 1990) with the cloned equine IgE CH3–CH4 fragment and repressor plasmid pREP4 were grown in 2 × YT medium supplemented with appropriate antibiotics at 37°C. Expression was induced at an OD₆₀₀ = 0.7–0.9 by addition of 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Boehringer-Mannheim, Mannheim, Germany). Incubation was continued for 4 h. The cells were then pelleted and lysed overnight at RT in 100 ml 6 M guanidine hydrochloride and 0.1 M NaH₂PO₄ (pH 8.0). The lysate was spun at 6000 × *g* for 10 min at 4°C and the supernatant applied to a column packed with 10 ml of Ni²⁺-chelate affinity resin (QIAGEN, Basel, Switzerland) at a flow rate of 1.1 ml/min. The recombinant equine IgE fragment (reqIgEf) was eluted with an 8 M urea step gradient (Hochuli et al., 1988). For in vitro refolding, the eluted recombinant protein was

dialysed twice against 0.266 mM acetic acid for 8 h at RT using a molecular-porous membrane with a 3.5 kDa cut-off (SPECTRA/POR, Socochim, Lausanne, Switzerland) followed by dialysis against distilled H₂O for 8 h. Protein concentration was determined using the BioRad protein assay with bovine serum albumin as a standard. The purity of the refolded protein was analysed by SDS-PAGE.

2.2. Induction of antibodies against the recombinant IgE fragment

Six laying hens (two Lohmann selected Leghorns and four Isabrown hybrids) were immunised up to seven times at intervals of two to three weeks by intramuscular injection of the immunogen in the pectoral region. The first injection contained 100–200 µg reqIgEf emulsified in complete Freund's Adjuvant (DIFCO Laboratories, Detroit, MI, USA) in a total volume of 0.5 ml. For the boosting injections the same quantity of immunogen was used, emulsified in incomplete Freund's Adjuvant. Eggs were collected pre- and postimmunisation. The egg yolks from the weekly production of each hen were pooled for immunoglobulin isolation. For this purpose the Eggstract™ IgY Purification System (Promega, Madison, WI, USA) was used according to the manufacturer's instructions. Two hundred to four hundred milligram protein per egg yolk was obtained. Analysis by SDS-PAGE showed that IgG was the main protein of this preparation.

One goat and two rabbits were immunised by subcutaneous injection of the same preparation. The rabbits were immunised twice and the goat eight times at intervals of two to three weeks. Sera were collected before immunisation and three weeks after each injection.

2.3. Characterisation of the raised antibodies with an ELISA

Immulon-2® polystyrene microtitre plates (Dynatech Laboratories, Chantilly, VA, USA) were coated at 4°C overnight with 100 µl of reqIgEf (1–100 ng/well) or with extracts of *Alternaria* (Trimedal, Brüttisellen, Switzerland) diluted 1:100 in 0.2 M sodium carbonate bicarbonate buffer, pH 9.4 (Pierce, Rockford, IL, USA). The coated plates were washed three times with 0.9% NaCl containing 0.25% Tween-20 (Fluka Chemie, Buchs, Switzerland). Unreacted protein-binding sites were subsequently blocked by incubation at 37°C for 2 h with 200 µl TBS pH 7.2 containing 0.2% (w/v) equine serum albumin (ESA) isolated from horse plasma according to Kistler and Nitschmann (1962). After washing the plates twice as above, serial twofold dilutions of anti-reqIgEf reagents (IgG from egg yolk) in TBS with 0.2% ESA and 1% Tween-20 were added to the wells (100 µl/well). The plates were then gently shaken at RT for 2 h. A rigorous washing followed: eight times as above and once with an increased concentration of 1% Tween-20. AP-labelled goat anti-chicken IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MA, USA) was diluted in TBS with 0.2% ESA and 1% Tween-20, and 100 µl was added to each well, followed by another incubation at RT on a shaker for 1 h. After washing the plates 4 × in 0.9% NaCl–0.25% Tween-20, 1 × in 0.9% NaCl and 1 × in H₂O, plates were developed by adding 100 µl/well of 10% diethanolamine buffer, pH 9.8 (Fluka Chemie) containing 0.5 mM MgCl₂ and 1 mg/ml *p*-nitrophenyl-phosphate (PNPP) (Sigma Chemical, St. Louis, MO, USA) as substrate. The plates were

incubated in darkness for 45 min at RT. The reaction was then stopped by addition of 50 μ l 4 M NaOH to each well and the optical density determined spectrophotometrically at 405 nm.

The ELISA for the detection of IgE antibody (Ab) in horse sera was carried out as above, but on the *Alternaria*-coated and ESA-blocked wells. One hundred μ l of horse sera diluted 1:4 to 1:16 in tris-buffered saline (TBS) with 0.1% ESA and 4% Tween-20 were added as 'first antibody.' Plates were incubated at room temperature (RT) on a shaker for 2.5–3 h and washed extensively. The anti-reqIgEf egg yolk IgG was then added in various dilutions and detected with alkaline phosphatase (AP)-labelled goat anti-chicken IgG. For IgG Ab detection in horse serum, the *Alternaria*-coated plates were blocked with 2% porcine skin gelatin for 2 h at 37°C and horse serum was then added at a dilution of 1:30. AP-labelled rabbit anti-horse IgG (Sigma) was diluted in the same buffer as used for IgE detection and 100 μ l was added to each well. Results were expressed as mean OD₄₀₅ of duplicate wells after subtraction of background values. The OD values of the negative controls varied between 0.06 and 0.10 for both IgE and IgG Abs; OD₄₀₅ values of 1.0 and above were accepted as positive reactions. For the comparison of the anti-reqIgEf IgG from different hens, the quantity of reagent needed to obtain an OD₄₀₅ of 1.0 was calculated in the linear titrable region of the reaction.

2.4. Antigen-affinity-chromatography enrichment of reqIgEf-specific antibodies

Recombinant equine IgEf was loaded on a Ni-NTA Superflow™ (Qiagen) column at a ratio of 2 mg protein per ml Ni-NTA resin by incubation at RT for 2 h on a shaker in TBS. After extensive washing with TBS (six times with 10 $\times$ volume of the matrix) the column was equilibrated with 'ImmunoPure gentle Ag/Ab binding buffer' pH 8.0 (Pierce). Five to ten milliliters (50–120 mg) of the IgG preparation isolated from the egg yolks of the immunised hens was added to the column (4 ml matrix) and incubated at RT on a shaker for 2 h. The column was washed six times with TBS and once with a 1 M NaCl solution. Bound immunoglobulins were eluted with 10 ml of 'Immunopure gentle Ag/Ab Elution buffer' (Pierce). ELISA analysis of the fractions collected (1.5 ml) using *Alternaria*-coated plates and horse serum showed that the specific anti-IgE antibodies were mainly eluted in the first fraction displaying an elevated protein content. The fractions containing the specific antibodies of different affinity-chromatography purification batches were pooled, desalted and concentrated by ultrafiltration with Centriplus® concentrators (Amicon, Beverly, MA, USA).

2.5. Antibody-affinity column for enrichment of horse IgE

Five milligrams of affinity-purified anti-reqIgEf IgG (see Section 2.4) were coupled to 3 ml Affi-Gel® Hz Hydrazide Gel (BioRad Lab) according to the manufacturers' instructions and packed into a column. Serum from a horse affected with severe summer dermatitis and COPD was precipitated with 45% saturated ammonium sulphate, resuspended in, and dialysed against, TBS pH 7.2. Ten milliliters of the immunoglobulin-rich precipitate were incubated with the column matrix for 2 h at RT. The column was then washed with four bed volumes of 1 M NaCl and with eight bed volumes of TBS pH 7.2.

Bound proteins were rapidly eluted with two bed volumes of 50 mM tris-HCl pH 2.6, containing 0.3 M glycine, and neutralised directly with 1 M tris-base, pH 11.2. The proteins recovered were concentrated by ultrafiltration with Centriplus® concentrators (Amicon) and analysed by SDS-PAGE and Western-blot.

2.6. Preparation of bronchoalveolar fluid

Bronchoalveolar lavage was performed according to Kleiber (1996) on a horse affected with COPD. After harvesting, the BALF was centrifuged at $400 \times g$ for 10 min. Two hundred and forty ml of the clear supernatant were precipitated with 50% ammonium sulphate for 30 min at RT and centrifuged at $12\,000 \times g$ for 10 min at 4°C. The pellet was resuspended in a minimum volume (1.8 ml) of PBS and extensively dialysed against PBS containing 0.5 M NaCl. The protein concentration of the BALF sample was 16 mg/ml.

2.7. Protein electrophoresis and immunoblotting

Recombinant equine IgEf, ammonium sulphate precipitated horse serum and BALF, IgE affinity enriched horse serum, and commercially obtained horse IgG (Sigma) and ESA (Sigma) (2–30 μ g per lane; 100 μ g per preparative gel) were separated by one dimensional SDS-PAGE on a vertical slab apparatus (MiniProtean II, BioRad) using 8 to 12% polyacrylamide gels run under denaturing-reducing or native conditions as described by Laemmli (1970) and Hames (1990), respectively. Staining with Coomassie brilliant blue G250 (Merck, Darmstadt, Germany) was carried out according to Towbin et al. (1979).

Transfer blotting of proteins from the polyacrylamide gel to nitro-cellulose membranes was performed according to Bjerrum and Schafer-Nielsen (1986) using semidry electrophoretic transfer (Multiphor II, Pharmacia, Uppsala, Sweden) at 70 mA for 1 h. The blots were blocked using 5% Nonfat dry milk (BioRad Lab.) in washing buffer (20 mM tris-HCl, 500 mM NaCl, 0.25% T, pH 7.5) and incubated for 2 h at RT, or overnight at 4°C with preimmune, purified chicken IgG or with reqIgEf-specific and affinity enriched chicken IgG. After washing three times the blot was incubated for 1 h with 0.1% AP-conjugated goat anti-chicken IgG (Kirkegaard and Perry Laboratories). Western-blots of the reqIgEf were also incubated with AP conjugate to Ni-NTA (QIAGEN). The blots were then washed and developed using 5-bromo-4-chloro-indolyl-phosphate (BCIP)/4-nitro blue tetrazolium chloride (NBT) (Boehringer-Mannheim) in AP buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris, pH 9.5).

2.8. Amino acid sequencing and analysis of a 80-kDa protein from horse serum affinity enriched for IgE

For amino acid sequencing and amino acid analysis, the IgE-enriched eluate was separated by one dimensional SDS-PAGE as described above, but subsequently blotted to Immobilon-P membrane (Millipore, Volketswil, Switzerland) according to Towbin et al. (1979), using a semidry transfer system (Pharmacia) run according to the manufac-

turer's manual. The transfer buffer contained 50 mM boric acid pH 9.0 and 20% methanol. The blot was stained with Ponceau S Solution (Sigma) and the 80 kDa band cut out before destaining.

N-terminal amino acid sequencing was carried out directly from the blotted protein using a pulsed-liquid-phase sequenator 477A from Applied Biosystems (Foster City, CA, USA). The released amino acids were analysed on-line according to instructions from Applied Biosystems.

For amino acid analysis the sample was hydrolysed in the gas-phase with 6 M hydrochloric acid containing 0.1% (by volume) phenol for 24 h at 115°C under vacuum according to Chang and Knecht (1991). The liberated amino acids were caused to react with phenylisothiocyanate and the resulting phenylthiocarbamyl amino acids analysed by reversed-phase high performance liquid chromatography on a Nova Pak C₁₈ column (Waters, Milford, MA, USA) in a Hewlett Packard liquid chromatograph 1090 with an automatic injection system according to Bidlingmeyer et al. (1984). The 0.14 M sodium acetate buffer, pH 6.4, was replaced by the corresponding ammonium acetate buffer.

2.9. Inhibition of the anti-reqIgEf reagent activity

To demonstrate the specificity of the reactions in the ELISA or in Western-blot, the reqIgEf-specific reagents were preincubated with increasing amounts (0.01–30 µg/ml) of reqIgEf, 0.03–30 µg/ml purified horse IgG (Sigma), 100 µg/ml nonrelevant [His]₆ proteins and *E. coli* lysate (100 µg/ml) for 2 h at RT.

Positive horse sera were heated at 54°C for 10 min before being used and compared to nonheated sera in ELISA.

2.10. Induction of reverse cutaneous anaphylaxis (RCA)

Two horses were prepared for intradermal injections by clipping an area of 25 × 15 cm on the shoulder. The area was cleaned and injection sites were marked with a pen to facilitate reading. The horses were tranquillised with xylazine (0.4 mg/kg body weight) and L-polamidon (0.05 mg/kg body weight). IgG isolated from eggs before immunisation or isolated and affinity purified after immunisation was diluted in physiological saline solution to the required concentrations. 100 and 30 µg of each in a volume of 50 µl were injected intradermally at separate sites with a 26 gauge needle in duplicates. As a further negative control, physiological saline solution was injected. Histamine dihydrochloride (8 µg/50 µl, Allergopharma J. Ganzer, Reinbek b. Hamburg, Germany) was used as a positive control.

The diameters of all wheals were measured after 0.5, 1.0 and 4.0 h.

3. Results

3.1. Expression and purification of a recombinant equine IgE fragment

The nucleotide sequence analysis of the insert in vector PDS781/RBSII, [His]₆-IgE CH3–CH4 was consistent with the original PCR product corresponding to these IgE Fc

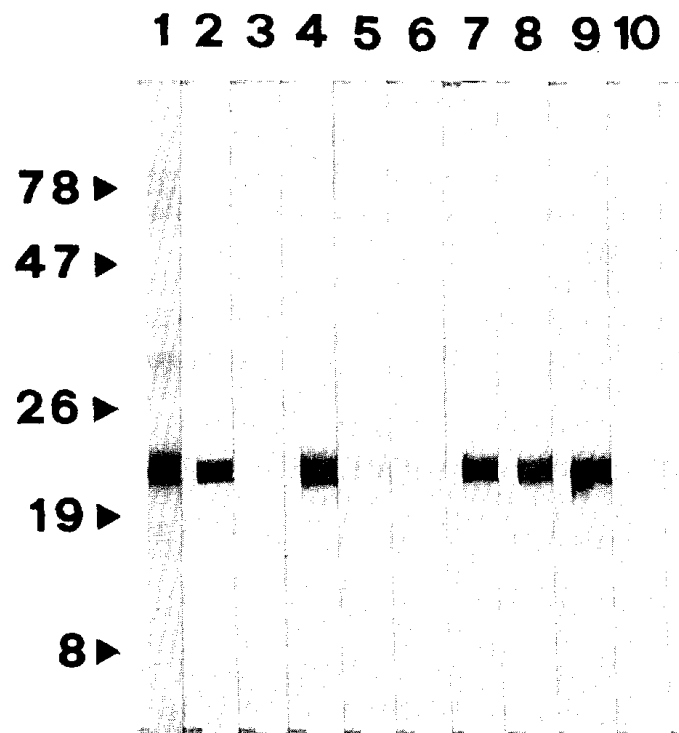


Fig. 1. Reducing 12% SDS-PAGE and Western-blot of the reqIgEf. Lane 1, SDS-PAGE stained with Coomassie blue. Western-blot incubated with: Lane 2, Ni-NTA AP conjugate; Lane 3, preimmune chicken IgG; Lane 4, anti-reqIgEf chicken IgG; Lane 5, inhibition of the anti-reqIgEf with 10 $\mu\text{g}/\text{ml}$ reqIgEf; Lane 6, inhibition with 100 $\mu\text{g}/\text{ml}$ reqIgEf; Lane 7, preincubation of anti-reqIgEf with 100 $\mu\text{g}/\text{ml}$ of a nonrelevant $[\text{His}]_6$ peptide; Lane 8, preincubation with 100 $\mu\text{g}/\text{ml}$ horse IgG; Lane 9, preincubation with 100 $\mu\text{g}/\text{ml}$ *E. coli* extract; Lane 10, conjugate control. Lanes 3–10 were visualised with an AP-labelled goat anti-chicken IgG. Molecular weights (in kDa) indicated at left.

domains. The yields of the $[\text{His}]_6$ -tagged fusion protein produced by induced *E. coli* M15 cells, after Ni^{2+} -chelate chromatography followed by refolding, were 5–10 mg/l of medium of induced cells. Twelve percent SDS-PAGE gel analysis of the purified reqIgEf under reducing conditions revealed one strong band (Fig. 1, lane 1) with a molecular mass of approximately 20.4 kDa, confirming the size of the fusion protein predicted from the nucleotide sequence (18.7 kDa). In addition, two minor, higher molecular weight contaminants were detected on the stained gel (Fig. 1, lane 1). However, the Ni-NTA AP conjugate reacted only with the major protein band of 20.4 kDa (Fig. 1, lane 2).

3.2. Reactivity of antibodies raised against reqIgEf

The reqIgEf fragment exhibited a strong immunogenic effect; 8 weeks after immunisation 0.16–0.29 $\mu\text{g}/\text{well}$ of IgG isolated from hen egg yolks reacted positively ($\text{OD} > 1.0$) in an ELISA against the solid phase bound reqIgEf (10 ng/well). The post immunisation chicken IgG recognised the 20.4 kDa reqIgEf protein in Western blot (Fig. 1). Preincubation of the immunoglobulin reagents with the reqIgEf (0.01–100 $\mu\text{g}/\text{ml}$)

resulted in a dose dependent inhibition of the reaction in ELISA and Western-blot. In contrast, *E. coli* lysate, equine IgG and a nonrelevant [His]₆-tagged protein had no inhibitory activities at doses up to 100 µg/ml as shown in Western-blot (Fig. 1).

Screening of 25 unselected horse sera (diluted 1:4 to 1:16) with the anti-reqIgEf egg yolk IgG on *Alternaria*-coated ELISA plates gave encouraging results in three of the six hens. Particularly, IgG from one hen (DB) displayed reproducible, positive results (OD ≥ 1.0) at concentrations of 6 µg/well with 8 horse sera. One of the negative controls (*Alternaria*, no horse serum) showed OD values of 0.12–0.18, suggesting a weak reaction of the chicken IgG with the coated allergen, whereas the other controls displayed OD values of 0.06–0.08. Further immunisations and experiments were only carried out with the three most promising hens (BB, DB, EB).

Preincubation of egg yolk antibodies with reqIgEf (3–10 µg/ml) resulted in a complete inhibition of the reaction.

3.3. Antigen-affinity chromatography

During the elution of the egg yolk antibodies from the reqIgEf–Ni²⁺-chelate column several fractions (1.5 ml) were collected and tested for their protein content and specific reactivity against horse serum in the '*Alternaria* ELISA'. Repeated experiments showed that the first fraction with increased protein content (0.4–1 mg/ml) always displayed a high specific activity. The eluate collected in the following fractions showed low or no specific activity despite a higher protein content.

Comparisons of the specific activity in relation to the protein content were made with IgG preparations before and after reqIgEf affinity chromatography. Amounts of immune protein required to achieve positive reactions (OD₄₀₅ = 1.0) were calculated and expressed in µg/well. ReqIgEf affinity chromatography resulted in a 1.8 to 4.4 activity increase of the reqIgEf-specific antibodies tested on the solid phase bound reqIgEf (Table 1). For the hen DB affinity chromatography resulted in a 27-fold higher specific activity on native horse IgE but in only a four-fold increased activity on reqIgEf (Table

Table 1

Amounts of immune IgG required to achieve positive reactions (OD₄₀₅ = 1.0) in ELISA tests on recombinant equine IgE fragment and on native horse IgE (*Alternaria*/horse serum ELISA). The negative controls displayed OD values of 0.06 to 0.12. Binding of IgG was measured with an anti-chicken IgG from the goat, conjugated to alkaline phosphatase and developed with the enzyme substrate PNPP

Source of IgG	Affinity purification	IgG in µg/well, required to obtain an OD = 1.0 in ELISA when tested on:	
		reqIgEf	<i>Alternaria</i> + horse serum
Eggs of hen BB	--	0.16	NA ^a
	+	0.09	0.6
Eggs of hen DB	--	0.29	5.9
	+	0.07	0.22
Eggs of hen EB	--	0.22	NA
	+	0.05	3.0

^aNA = not applicable, IgY reacted with *Alternaria* before reqIgEf affinity-chromatography and thus could not be used in the '*Alternaria*/horse serum' ELISA.

1). Hens BB and EB had a high natural antibody response against *Alternaria* and could thus not be used in the *Alternaria*/horse serum ELISA before affinity purification.

Compared to the IgG preparation from the egg yolks, the reqIgEf affinity-purified reagents no longer showed any reactivity against allergens (*A. fumigatus*, *Alternaria*, mites extracts, etc.) when tested in ELISA. The reqIgEf affinity column is very stable and the binding capacity did not decrease even after having been used more than 20 times.

3.4. Inhibition of the anti-reqIgEf reagent activity

The following experiments show in the '*Alternaria*/horse serum' ELISA that the developed reagent specifically recognises horse IgE.

3.4.1. Inhibition with reqIgEf

Incubation of the immune and reqIgEf affinity-purified chicken IgG with different concentrations (10–0.01 $\mu\text{g}/\text{ml}$) of reqIgEf resulted in a dose dependent inhibition of the reaction (Table 2). With 10 $\mu\text{g}/\text{ml}$ a complete inhibition was achieved. After incubation with 0.1 $\mu\text{g}/\text{ml}$ reqIgEf only 50% of the OD value obtained without inhibition was reached. No inhibition was observed after incubation of the reagent with horse IgG in different concentrations. IgG detection was, however, strongly inhibited by

Table 2

Detection of *Alternaria*-specific IgE and IgG in sera from two clinically healthy horses in an ELISA, and inhibition of the reactions by preincubation of the anti-reqIgEf chicken IgY with reqIgEf and horse IgG and heat inactivation of IgE. Sera were diluted 1:6 for IgE and 1:30 for IgG detection. IgG was detected with an AP-labelled rabbit anti-horse IgG

	Detection of IgE		Detection of IgG	
	Horse 1	Horse 2	Horse 1	Horse 2
No inhibition:	100 ^a (OD ₄₀₅ = 0.85)	100 (OD ₄₀₅ = 1.32)	100 (OD ₄₀₅ = 1.13)	100 (OD ₄₀₅ = 1.30)
Inhibition with: <i>reqIgEf</i>				
10 $\mu\text{g}/\text{ml}$	7	6	86	79
1 $\mu\text{g}/\text{ml}$	13	10	74	74
0.1 $\mu\text{g}/\text{ml}$	54	52	ND	ND
0.01 $\mu\text{g}/\text{ml}$	99	97	ND	ND
<i>IgG</i>				
30 $\mu\text{g}/\text{ml}$	112	92	4	5
3 $\mu\text{g}/\text{ml}$	100	90	19	18
0.3 $\mu\text{g}/\text{ml}$	98	91	49	51
0.03 $\mu\text{g}/\text{ml}$	102	92	68	67
Serum heated 10 min at 54°C	0	0	100	100

^a Values shown in table are mean OD₄₀₅ of duplicate wells after subtraction of background values (0.08–0.15) and expressed in % adsorbance of the values obtained without inhibition.

ND = not done.

preincubation of the anti-horse IgG with the same IgG preparation as used above, but was not influenced by preincubation with reqIgEf (Table 2).

3.4.2. Preincubation with equine colostrum

In a pilot experiment, a possible inhibitory activity of equine colostrum (rich in IgG and IgA) on the anti-reqIgEf chicken IgG was tested by preincubation of the chicken IgG with an end concentration of 2% colostrum. The reaction in the *Alternaria*/horse serum ELISA was not inhibited.

3.4.3. Heat inactivation

Heating positive horse sera at 54°C for 10 min before adding them to the allergen-coated wells diminished drastically the recognition by the immune, affinity-purified chicken IgG antibodies to 5–10% of the OD₄₀₅ value of the same unheated horse sera (Table 2). IgG titres remained unchanged after the heat treatment.

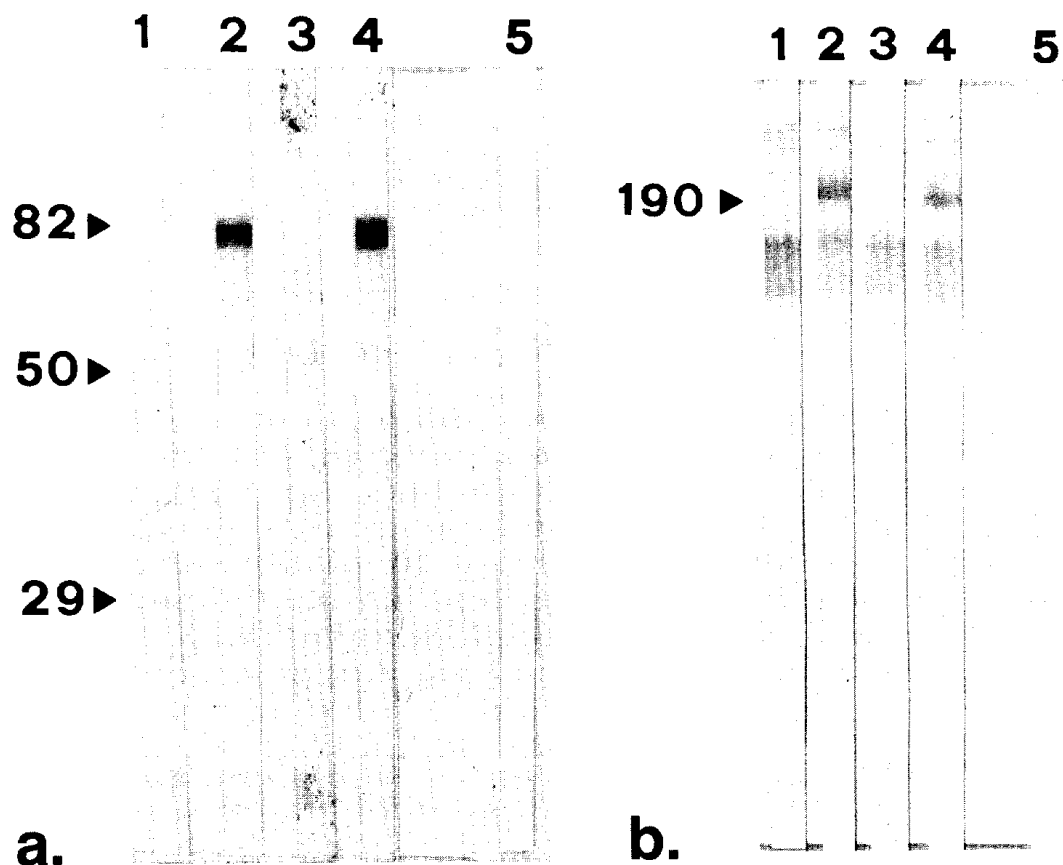


Fig. 2. SDS-PAGE Western-blot of ammonium sulphate precipitated serum of a horse. (a) 10% SDS-PAGE under reducing conditions. (b) 8% SDS-PAGE under nonreducing conditions. The blot was incubated with: Lane 1, preimmune chicken IgG; Lane 2, anti-reqIgEf chicken IgG; Lane 3, anti-reqIgEf, preincubated with 3 µg/ml reqIgEf; Lane 4, anti-reqIgEf, preincubated with 30 µg/ml horse IgG; Lane 5, conjugate control. The blot was visualised using an AP-labelled goat anti-chicken IgG. Molecular weights (in kDa) indicated at left.

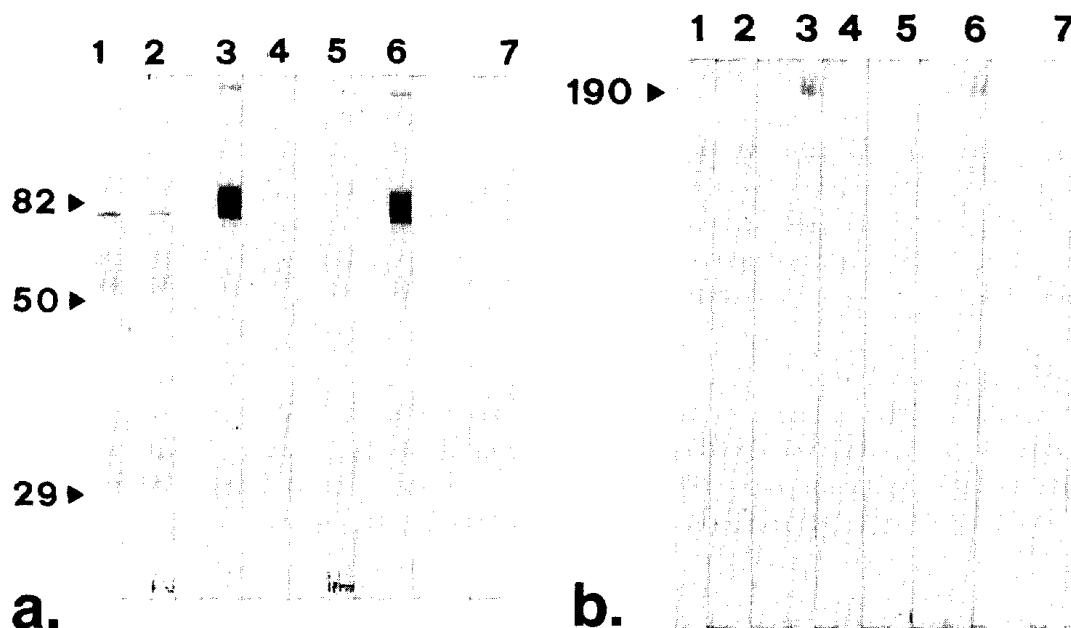
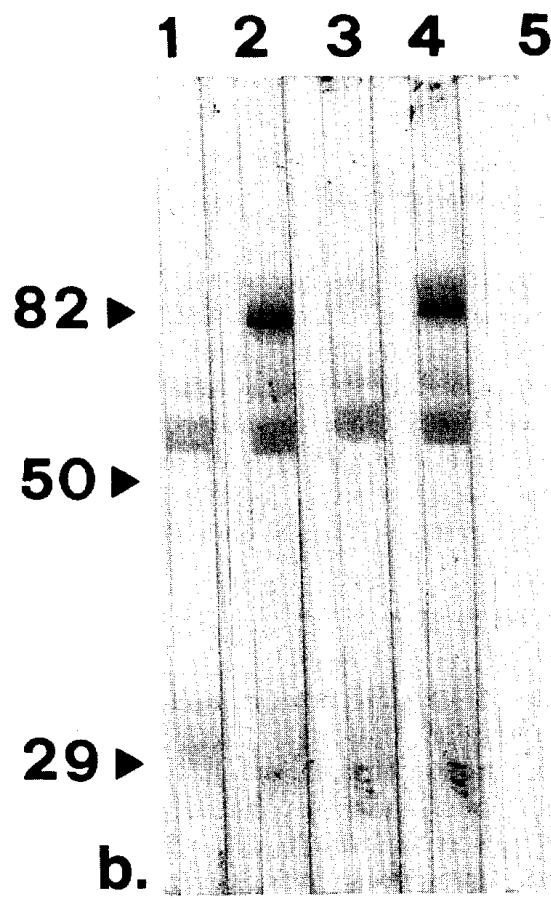
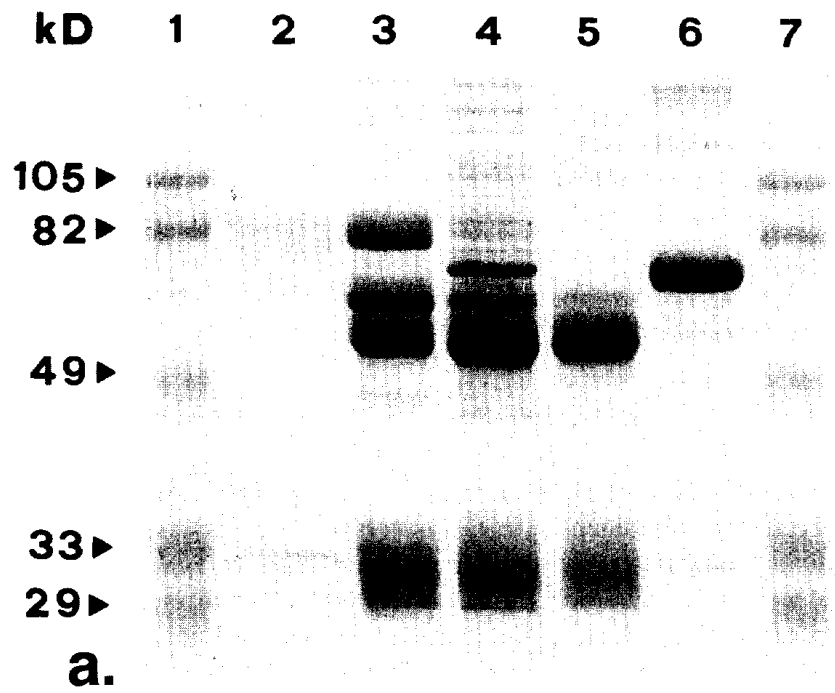


Fig. 3. SDS-PAGE Western-blot of ammonium sulphate precipitated bronchioalveolar fluid of a horse. (a) 10% SDS-PAGE under reducing conditions. (b) 8% SDS-PAGE under nonreducing conditions. The blot was incubated with: Lane 1, preimmune chicken IgG; Lane 2, preimmune chicken IgG, preincubated with 3 $\mu\text{g}/\text{ml}$ reqIgEf; Lane 3, anti-reqIgEf chicken IgG; Lane 4, anti-reqIgEf, preincubated with 3 $\mu\text{g}/\text{ml}$ reqIgEf; Lane 5, anti-reqIgEf, preincubated with 30 $\mu\text{g}/\text{ml}$ reqIgEf; Lane 6, anti-reqIgEf, preincubated with 30 $\mu\text{g}/\text{ml}$ horse IgG; Lane 7, conjugate control. The blot was visualised using an AP-labelled goat anti-chicken IgG. Molecular weights (in kDa) indicated at left.

3.5. Reaction of antibodies in immunoblots

The immunoblotting assays of ammonium sulphate precipitated proteins from horse sera and BALF were carried out under denaturing–reducing (SDS-10% PAGE) and nonreducing (SDS-8% PAGE) conditions. As illustrated in Figs. 2 and 3, the anti-reqIgEf-specific IgG reacted under reducing conditions with the electrophoretically separated precipitates of serum or BALF at 78–80 kDa. This molecular weight corresponds to the expected size of the heavy chains of horse IgE molecules. The same precipitates separated under nonreducing condition displayed a reaction at a molecular weight of 190–200 kDa, corresponding to the estimated size of the whole equine IgE. The

Fig. 4. (a) Affinity chromatographic enrichment of equine IgE from ammonium sulphate precipitated serum. 10% SDS-PAGE under reducing conditions, with Coomassie Blue stain. Lanes 1 and 7, molecular weight standards. Lane 2, human myeloma IgE. Lane 3, equine IgE-enriched eluate from the affinity-chromatography column. Lane 4, ammonium sulphate precipitated serum before affinity-chromatography. Lanes 5 and 6, commercially obtained horse IgG and equine serum albumin, respectively. (b) SDS-PAGE Western-blot of affinity enriched horse IgE (Lane 3 in (a)). The blot was incubated with: Lane 1, preimmune chicken IgG. Lane 2, anti-reqIgEf chicken IgG. Lane 3, anti-reqIgEf preincubated with 3 $\mu\text{g}/\text{ml}$ reqIgEf. Lane 4, anti-reqIgEf preincubated with 30 $\mu\text{g}/\text{ml}$ horse IgG. Lane 5, conjugate control. The blot was visualised using an AP-labelled goat anti-chicken IgG. Molecular weights (in kDa) indicated at left.



reactions observed under nonreducing and reducing conditions were completely inhibited by preincubation of the immune chicken IgG with 3 $\mu\text{g}/\text{ml}$ reqIgEf. No inhibition however, was achieved by preincubation of the reqIgEf-specific chicken IgG with horse IgG at a concentration of 30 $\mu\text{g}/\text{ml}$. Preimmune chicken IgG did not show any reaction at these respective molecular weights.

The reqIgEf-specific antisera from the two rabbits and the goat reacted with high titres (1:20 000) against the reqIgEf and also recognised the 75–80 kDa protein in denaturing–reducing Western-blot of ammonium sulphate precipitated horse serum and BALF. However, they did not react with the native horse IgE as shown by Western-blot under unreduced conditions (data not shown).

3.6. Affinity enrichment of horse serum IgE with anti-reqIgEf chicken antibodies

SDS 10% PAGE analysis of the eluate from the anti-reqIgEf affinity chromatography column shows a strong enrichment of the 80 kDa band (Fig. 4a, lane 3) compared to the ammonium sulphate precipitated serum before affinity chromatography (Fig. 4a, lane 4). The molecular weight of the enriched band is similar to human myeloma IgE (Fig. 4a, lane 2). Comparison of the eluate with commercially obtained horse IgG (Fig. 4a, lane 5) shows that there appears to be some contamination of the eluate with horse IgG. The reqIgEf-specific chicken IgG only reacts with the 80 kDa band in immunoblot of the IgE enriched eluate (Fig. 4b, lane 2). This reaction is inhibitable by preincubation of the anti-reqIgEf antibodies with reqIgEf (Fig. 4b, lane 3) but is not inhibited by preincubation with horse IgG (Fig. 4b, lane 4).

3.7. Amino acid sequencing and analysis

No sequence could be obtained by N-terminal analysis. The amount of protein present on the membrane was indicative for a blocked N-terminus, therefore we resolved to use amino acid analysis for protein identification.

The amino acid composition of the isolated 80 kDa band was in good agreement with the calculated values from the deduced amino acid sequence of the equine heavy chain published previously by Navarro et al. (1995) as shown in Table 3. The results represent uncorrected data after gas-phase hydrolysis. The elevated glycine content is due to the presence of this amino acid in the buffer used during SDS-PAGE procedure.

3.8. Reverse cutaneous anaphylaxis

Intradermal injections of 100 and 30 μg affinity-purified reqIgEf-specific IgG resulted after 30 min in reactions (horizontal diameter of the wheals 13 and 15 mm, respectively) which were stronger than the negative controls (0.9% NaCl and same concentrations of preimmune IgG, horizontal diameter of 10 mm) but smaller than the wheals elicited by 8 μg histamine dihydrochloride (wheal of 20 mm). Most remarkably, the thickness of the wheals provoked by the reqIgEf-specific reagent were similar to

Table 3

Amino acid composition of the 80 kDa serum protein detected by immunoblot of the reqIgEf-specific chicken antibody. The results represent uncorrected data after gas-phase hydrolysis with 6 M hydrochloric acid. The expected values are calculated from the published amino acid sequence of the equine IgE heavy chain (Navarro et al., 1995)

Amino acid	Obtained	Expected
Asp	47.2	45
Glu	66.0	72
Ser	55.9	52
Gly	52.9	34
His	11.5	9
Arg	22.2	18
Thr	40.7	51
Ala	28.5	33
Pro	41.5	45
Tyr	16.2	18
Val	46.5	48
Met	7.2	3
C–C	ND	ND
Ile	17.5	18
Leu	44.6	48
Phe	19.1	20
Lys	32.2	36
Trp	ND	ND

ND = not done.

those raised by histamine (4–6 mm) and distinctly higher than the negative controls (wheals flat, 1–2 mm). The wheals started to decrease slowly after 2 h. The RCA was carried out on two horses with similar results.

4. Discussion

The aim of the present investigation was to produce immunoreagent(s) specific for equine IgE. In a previous study, Chrétien et al. (1988) demonstrated that some IgE heavy chain-specific monoclonal antibodies produced by immunisation with human IgE myeloma proteins also reacted with recombinant IgE fragments expressed in *E. coli*. These observations encouraged us to try the reverse experiment in the horse, using a recombinant horse IgE fragment for immunisations and hypothesising that the raised antibodies would also react with the native IgE molecules. As shown in the results, the reqIgE ϵ -chain fragment (nucleotides 1132 to 1592) elicited antibodies with high titres in all immunised hens. The reaction was detectable not only with the immunogen but also with immunoglobulin enriched precipitates from horse serum and BALF in Western-blot analysis under reducing as well as nonreducing conditions. The reaction with a 75–80 kDa or 190–200 kDa protein, respectively, and the inhibition by preincubation with the reqIgEf suggested strongly that the produced antibodies recognise the equine IgE heavy chain in reduced and also in unreduced form. The immunised goat and rabbits, however, only reacted with the reduced equine IgE heavy chain.

The same was true when horse sera were tested in an ELISA with allergens bound to the solid phase: only the immunoreagents from some chickens gave true positive reactions with some of the horse sera tested. The inhibition experiments with reqIgEf, horse IgG, horse colostrum and with nonrelevant [His]₆-tagged polypeptides, as well as the heat lability of the detected immunoglobulin, suggest again that the target molecule is horse IgE. Heat treatment of IgE affects not only its capacity for passive sensitisation, i.e. its binding capacity to mast cells and basophils, but also binding of anti-IgE antibodies to IgE (Ishizaka et al., 1967).

Enrichment of the 75–80 kDa band from ammonium sulphate precipitated serum by the immune chicken antibody-affinity column allowed further analysis of this protein by determination of its amino acid composition. N-terminal analysis revealed a blocked N-terminus. However, the amino acid analysis strongly indicates that the 80 kDa band corresponds to the heavy chain of horse IgE. The small differences between the determined/expected amino acid composition of the equine IgE heavy chain are probably of a technical nature, like for example the elevated glycine content of the sample which was due to the presence of glycine in the buffer during SDS-PAGE. Furthermore, subclass or allotypic variations could also cause small differences.

The IgG contamination after affinity enrichment for horse IgE (Fig. 4a) is not due to cross-reactivity of the anti-reqIgEf with horse IgG, but can perhaps be explained by copurification of IgE–IgG autoantibody complexes present in the sera (Fiebriger et al., 1996). Furthermore, most horses have IgG and IgE antibodies against chicken IgG as shown in an ELISA with chicken IgG bound to the solid phase (data not shown). This is easily explained by the fact that nearly all horses are immunised against influenza A/equi 1 and equi 2 and that the virus used is produced in eggs.

The reverse cutaneous anaphylaxis gave, compared to histamine, weak but consistent results. In both tested horses the specific and affinity-chromatography enriched IgG raised against reqIgEf gave stronger reactions than the preimmune IgG used as a negative control.

All results suggest that the reqIgEf has at least two antigenic determinants. One or more of them elicit antibodies in all species and they recognise the IgE heavy chain in reduced form. These epitope(s) are likely to represent linear epitopes and do not recognise the folded native IgE antibodies. The other determinant(s), present on a heat labile region of the native IgE molecule, induced antibodies only in some hens but not in any of the mammals used for immunisation. This suggests that this epitope(s) may represent a conserved region of the IgE heavy chain, only immunogenic in birds which are phylogenetically more distant from the horse than other mammals. Immunisation of laying hens thus turned out to be the most efficient way to produce antibodies recognising the native horse IgE. However, not every hen produced antibodies against the critical antigenic determinant of reqIgEf able to recognise native horse IgE. This limitation is compensated by the large quantity of specific IgG that can be isolated from the egg yolks produced by one single hen.

The relative enrichment of the activity against native IgE compared to the activity against reqIgEf during the reqIgEf antigen affinity chromatography (27 times higher activity when tested on native IgE versus four times higher activity when tested on reqIgEf in ELISA) suggests that even in chickens, antibodies against the epitope present

on native IgE occur in a much lower proportion than those against the determinant(s) present on the reqIgEf. These two determinants are specific for the heavy chain of IgE as demonstrated by Western-blot and the lack of inhibition of the reaction by high doses of IgG.

The horse IgE molecule seems to be more sensitive to heat treatment than the IgE of other species, where inactivation is described after a 30-min to 4-h incubation at 56°C (Ishizaka et al., 1967). Incubation of horse serum at 54°C for 10 min resulted in 90–95% loss of the binding activity of the anti-reqIgEf antibodies on heated serum IgE compared to unheated serum.

Binding of the [His]₆-tagged recombinant protein to the Ni-chelate column was an excellent tool for the affinity purification of the egg yolk IgG. The raw IgG isolates showed some unspecific binding, furthermore they contained antibodies against environmental allergens, like for example *A. fumigatus* or *Alternaria*. With the reqIgEf affinity chromatography all the undesirable features of the raw IgG preparations were eliminated. Similar results were obtained by Gu et al. (1994) using the same method with another [His]₆ recombinant protein for affinity purification of a rabbit antiserum. This technique can be recommended for purification of antibodies against recombinant proteins containing the [His]₆ tag because of the limited quantity of protein needed and the simplicity and effectiveness of the method.

The herein presented investigation resulted in the development of polyclonal egg yolk antibodies specific for equine IgE heavy chain. These reagents will permit to carry out studies on many aspects of allergic diseases in the horse.

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