

Expression of a 4-(hydroxy-3-nitro-phenyl) acetyl (NP) specific equi-murine IgE antibody that mediates histamine release *in vitro* and a *type I* skin reaction *in vivo*

B. WAGNER*, G. SIEBENKOTTEN[†], W. LEIBOLD and A. RADBRUCH[†]

Immunology, School of Veterinary Medicine, Bischofsholer Damm 15, 30173 Hannover and [†]German Rheumatism Research Center, Schumann Str. 21/22, 10117 Berlin, Germany.

Keywords: horse; equine IgE; recombinant proteins; transfectomas; biological activity; N-glycosilation

Abbreviations

BSA: bovine serum albumin
ELISA: enzyme linked immunosorbent assay
FcεRI: high affinity receptor for IgE
2-ME: 2-mercaptoethanol
mab = monoclonal antibody
NIP: 4-(hydroxy-3-iodo-5-nitro-phenyl) acetyl
NP: 4-(hydroxy-3-nitro-phenyl) acetyl
PBS: phosphate-buffered saline
NP-IgE: NP-specific IgE (IgE anti NP)
PCR: polymerase chain reaction
SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis
V_H: variable region of the immunoglobulin heavy chain

Summary

Due to characteristic clinical signs, immunoglobulins of isotype E (IgE) are believed to be involved in several allergic diseases of the horse. To date, closer investigations have been hampered by the fact that neither purified equine IgE nor anti-equine IgE monoclonal antibodies were available for IgE isotype determination. As an approach to solve this problem, we constructed a stable cell line (EqE6) that expresses recombinant equi-murine IgE specific for 4-(hydroxy-3-nitro-phenyl) acetyl (NP). Biochemical analysis of the purified protein revealed a highly glycosilated IgE monomer of approximately 230,000 Da. The biological ability of the NP-IgE to mediate histamine release after crosslinking with antigen was demonstrated *in vitro* using equine blood leucocytes. *In vivo*, the intradermal application of NP-IgE followed by antigen crosslinking induced a *type I* hypersensitivity skin reaction in horses. Both results indicate that the recombinant NP-IgE contains an intact and functional FcεRI binding site and mediates effector functions in equine basophils and cutaneous mast cells. This equi-murine IgE can be used for the production of IgE-specific polyclonal and monoclonal antibodies. In addition, the NP specificity allows the antigen-specific activation of equine Fcε-receptor-

expressing cells, such as mast cells and basophils. This property could be used to investigate IgE-mediated mechanisms for a better understanding of equine *type I* allergic diseases.

Introduction

Equine IgE is believed to be involved in parasitic infections (Suter and Fey 1981) and in *type I*-mediated allergic diseases (Matthews *et al.* 1983). Due to their clinical signs, IgE-mediated mechanisms seem to play a role in many equine diseases, such as chronic, obstructive pulmonary disease (McGorum *et al.* 1993; Schmallenbach *et al.* 1998), sweet itch (Larsen *et al.* 1988; Ungar-Waron *et al.* 1990) urticaria or head-shaking (Mansfield *et al.* 1998). While reagents against IgE are readily available for other species, determination of equine IgE is hampered by the lack of detection antibodies and pure reference proteins.

Although partial purification of IgE from equine serum has been attempted, these samples were still contaminated with a substantial amount of other immunoglobulin isotypes (Suter and Fey 1981). In addition, myeloma cell lines secreting pure horse IgE are not available. Therefore, the generation of recombinant equine IgE could be used as reference protein and for the production of equine IgE-specific antibodies. While recombinant bovine (Knight *et al.* 1988) and ovine (Clarke and Beh 1994) IgE have been expressed successfully in different eukaryotic systems, to date only the expression of incomplete equine IgE heavy chain fragments in *E.coli* and the production of polyclonal equine IgE-specific antibodies using these recombinant proteins have been described (Marti *et al.* 1997; Watson *et al.* 1997).

We constructed a new cell line by transfecting J558L cells with an expression vector containing the cDNA for the equine Cε constant heavy chain region and the murine V_H186.2 gene. The secreted protein was purified by immunoaffinity chromatography taking advantage of its specificity for the NP antigen. Therefore, for the first time, it was possible to produce, purify and characterise a biologically active NP-specific equi-murine IgE in mammalian cells, containing the complete equine Cε heavy chain region.

Materials and methods

Animal studies were performed following the recommendations of the German Animal Welfare Act.

*Author to whom correspondence should be addressed.

Synthesis of cDNA of the murine VH186.2 and the equine Cε gene

The total RNA from the murine NP-specific IgD producing myeloma cell line B1-8δ (Neuberger and Rajewsky 1981), and from equine peripheral blood mononuclear cells of one horse (Wagner *et al.* 2001) were isolated using the RNeasy Mini Kit¹. The cDNA was generated from RNA by 1st strand reverse transcriptase reaction using the oligo(dT)₁₅ primer² and SuperScript II reverse transcriptase³, following the manufacturer's instructions.

Selection of primers for PCR

The murine VH186.2 cDNA was amplified by PCR with the following primers, which were designed using the EMBL nucleotide databank sequence J00529 (Bothwell *et al.* 1981):

5'TCGAGCGGCCGCCAGCCATGGGATGGAGCTGT 3' -

corresponding to the 5' end of the VH186.2 signal peptide sequence and containing a *Not I* site and a Kozak sequence (Kozak 1989) for cloning and efficient protein expression, and:

5'GGCCGAATTCCTGAGGAGACTGTGAGAGTGG 3' -

representing the antisense sequence of the 3' end of the VH186.2 gene and containing an *EcoRI* site for cloning.

For amplification of the complete equine Cε cDNA, 2 primers were designed according to the published sequence U15150 (Navarro *et al.* 1995):

5' GTCTCCAAGCAAGCCCCATTA 3' -

corresponding to the 5' sequence of the equine Cε1 exon, and:

5' TCGCAAGCTTTACCAGGGTCTTTGGACACCTC 3' -

corresponding to the antisense sequence of the 3' end of the equine Cε4 exon. The Cε4 primer contains a *Hind III* site for cloning the PCR product.

PCR protocol

The PCR was performed using 1 µl of each cDNA as a template and a final concentration of 4 mmol/l MgCl₂, 200 µmol of dNTPs² and 0.2 nmol of each primer³ in 1x Pfu DNA polymerase reaction buffer². The PCR reaction was carried out in a Personal Cycler⁴ using the following cycling programme: 1) 4 min 94°C; 2) 1 min 84°C; 3) 1 min 50°C; 4) 3 min 72°C; 5) 1 min 94°C; 6) 1 min 50°C; 7) 3 min 72°C with 28 repeats of steps 5–7), and 8) 7 min 72°C. The PCR reaction was started by adding 1.25 u Pfu DNA polymerase during step 2.

At the end of the reaction, 2.5 u Taq DNA polymerase³ were added on ice, mixed and incubated for an additional 10 min at 72°C for addition of 3' A-overhangs. The PCR products were purified by one phenol/chloroform extraction step according to standard protocols (Sambrook *et al.* 1989), cloned into the pCRTMII vector⁵ and sequenced by automatic sequencing⁶. The M13rev and T7 promotor standard primers were used for sequencing of the murine VH186.2 gene. The equine Cε gene was sequenced as described previously (Wagner *et al.* 2001).

Cloning of the VH186.2 and Cε cDNA into the expression vector

The murine VH186.2 and the equine Cε cDNA (Cε^b, AJ305048) were isolated from the pCRTMII vectors by *NotI/EcoRI* or *EcoRI/Hind III* restriction, respectively, and cloned sequentially in the mammalian expression vector pcDNA3.1(-)Myc-His⁵ in frame

with the c-terminal myc-epitope and His₆ residue. The VH186.2-Cε expression vector contains a neomycin selection marker, mediating resistance for the aminoglycosidic antibiotic G418.

J558L cells

Murine J558L myeloma cells (Oi *et al.* 1983), producing λ₁ light chains were used for transfection. Cells were maintained in cell culture medium (RPMI 1640)⁷, supplemented with 2 mmol/l L-glutamine, 18 mmol/l NaHCO₃, 100 iu/ml penicillin, 100 µg/ml streptomycin⁸ and 10% (v/v) heat-treated (56°C, 60 min) fetal calf serum⁹.

Transfection and G418-selection

For transfection, 1 x 10⁷ J558L cells were washed once in transfection medium (RPMI 1640, supplemented as described above, but without fetal calf serum). The cells and 20 µg of the *Pvu I* linearised VH186.2-Cε expression vector were transferred in a total volume of 300 µl transfection medium into a 0.4 cm electroporation cuvette¹⁰. The cuvette was placed on ice for 9 min and at room temperature for 1 min. Cells were then transfected by electroporation at 270 V and 960 µF using a Gene Pulser¹⁰. Subsequently, the electroporation cuvette was placed in a 37°C incubator for 10 min before the cells were transferred to a cell culture plate (ø 9 cm), containing prewarmed cell culture medium (see above).

After 24 h, an aliquot of the transfectants was tested by intracellular staining and the resting cells were plated out in cell culture medium, containing 1 µg/ml G418⁵ to select all cells which had stably incorporated the transfected DNA.

Intracellular staining

Approximately 2 x 10⁶ transfected cells were washed once with PBS (Biochrom) and fixed in 2% (v/v) formaldehyde¹¹ for 20 min. Cells were washed twice with PBS (100 x g, 5 min) and subsequently stained with the murine monoclonal antibody Ac146, recognising the VH186.2 domain (Reth *et al.* 1979). The Ac146 antibody was diluted to a final concentration of 10 µg/ml in saponin buffer (PBS, supplemented with 0.5% [w/v] bovine serum albumin [BSA]¹¹, 0.5% [w/v] saponin¹² and 0.02% [w/v] NaN₃¹²) and cells were incubated for 30 min. Cells were then washed twice in saponin buffer and stained with a fluorescein conjugated goat anti mouse IgG + IgM (H+L) antibody¹³ diluted 1:50 in the same buffer for 20 min. Cells were washed twice more in saponin buffer, resuspended in PBS and evaluated by FACScan¹⁴. All incubation and washing steps were performed at room temperature.

Enzyme linked immunosorbent assay (ELISA)

Microtitre plates¹⁵ were coated with 100 µl/well of the NP derivative 4-(hydroxy-3-iodo-5-nitro-phenyl) acetyl (NIP) coupled to BSA (NIP₁₅-BSA)¹⁶ in a final concentration of 5 µg/ml in carbonate buffer (15 mmol/l Na₂CO₃, 35 mmol/l NaHCO₃, pH 9.6) and incubated overnight at 4°C. Plates were washed 5 times with phosphate buffer (2.5 mmol/l NaH₂PO₄, 7.5 mmol/l Na₂HPO₄, 145 mmol/l NaCl, 0.1% [v/v] Tween 20, pH 7.2). Cell culture supernatants containing the NP-specific proteins were diluted 1:5 in phosphate buffer (50 µl/well) and incubated for 1 h

on a plate rotator. After 5 washes (see above), 50 μ l of the mouse λ chain specific monoclonal antibody Ls 136 (Reth *et al.* 1979) was added to each well in a concentration of 1 μ g/ml in phosphate buffer and incubated for 1 h. Plates were washed, as described above, and peroxidase conjugated goat anti mouse IgG (H+L)¹³ was added in a dilution of 1:20,000 in phosphate buffer (50 μ l/well) and incubated for 45 min. Plates were washed once more and, subsequently, 50 μ l of substrate buffer were added (33.3 mmol/l citric acid, 66.7 mmol/l NaH₂PO₄, pH 5.0), supplemented with 130 μ g/ml 3,3',5,5'-tetramethylbenzidine (TMB)¹² and 0.01% (v/v) hydrogen peroxide¹². Incubation was for 20 min in the dark and the reaction was stopped by adding one volume of 0.5 mol H₂SO₄. Plates were evaluated in an ELISA reader¹⁷ at 450 nm and 630 nm absorbance. Dilutions of purified NP-specific murine IgD were used as positive controls and for quantification of NP-specific equine IgE.

Purification of NP-specific IgE

NP-specific equine IgE (NP-IgE) was purified from cell culture supernatant by immunoaffinity chromatography using NP coupled CNBr-activated sepharose 4B¹⁸. After binding, the column was washed with PBS. The NP-IgE was eluted with 100 mmol/l glycine-HCl (pH 3.0) and the fraction was collected in a tube containing 1 mol/l Tris-HCl solution (pH 7.0) for neutralisation. The eluted protein was dialysed overnight against PBS and concentrated using an Ultrafree15 concentrator¹⁹.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Approximately 1 μ g NP-IgE was incubated with 0.5 u N-glycosidase F²⁰ in a total volume of 15 μ l sodium phosphate buffer (160 mmol/l, pH 7.0). Glycosidase treatment was performed overnight at 37°C. As a control, a protein sample was incubated in sodium phosphate buffer without N-glycosidase F. Before samples were loaded onto the gel, 15 μ l of double concentrated SDS-buffer (125 mmol/l Tris, 20% [v/v] glycerol, 4% [w/v] SDS, 1% [w/v] bromophenol blue, pH 6.8), containing 10% (v/v) 2-mercaptoethanol (2-ME) were added and the samples were boiled for 5 min. Alternatively, the sample was boiled in SDS-buffer without 2-ME. SDS-PAGE and Western blotting were performed as described previously (Wagner *et al.* 1995).

Proteins were detected either by Coomassie brilliant blue staining of the SDS-PAGE gel (Sambrook *et al.* 1989) or by immunoblotting (Wagner *et al.* 1995) using murine monoclonal antibodies specific for the myc epitope⁵ or Ac 38 which is specific for the NP recognition site (Reth *et al.* 1979), and an alkaline phosphatase conjugated goat anti-mouse IgG (H+L) secondary antibody¹³.

Isolation of equine peripheral blood leucocytes

Equine leucocytes were isolated from 10 ml of heparinised peripheral blood of 2 foals (age 7 days and 2.5 months). Blood samples were allowed to sediment for approximately 30 min until the red blood cells were clearly separated from the plasma enriched with leucocytes. The leucocyte-enriched plasma was transferred carefully to a new tube and centrifuged (100 g, 5 min, room temperature). The plasma was removed and the cells were washed twice in PBS. The leucocytes were then resuspended in

release buffer (119 mmol/l NaCl, 5 mmol/l KCl, 0.4 mmol/l MgSO₄, 25 mmol/l Pipes acid, 56 mmol/l glucose, 40 mmol/l NaOH; shortly before use 1 mmol/l CaCl₂ and 0.1 % [v/v] autologous equine plasma were added).

Histamine release from equine leucocytes

Increasing concentrations of purified NP-IgE (0.3125, 0.625, 1.25 and 2.5 μ g/ml) were added to aliquots of approximately 1 x 10⁶ equine leucocytes in a total volume of 50 μ l release buffer. Leucocytes of young foals were used, because we expected that the occurrence of free Fc ϵ -receptors on blood basophils was more likely than on cells of mature horses. The cells were incubated for 2.5 h in a CO₂ incubator at 37°C, washed with 1 ml of release buffer and incubated for an additional hour (CO₂, 37°C) with NIP₁₅-BSA(25 μ g/ml) diluted in 100 μ l release buffer. The tubes were then centrifuged (100 g, 5 min, room temperature) and the supernatants were collected for histamine detection.

Histamine concentrations were determined by a competitive radioimmunoassay; to 100 μ l supernatant from the histamine release test, 50 μ l Tris buffer (500 mmol/l Tris, 0.02% [v/v] Tween 20, pH 8.5) and 50 μ l acylating reagent (2 mg/ml NHS-Biotin²¹ in 110 mmol/l NaCl, 5 mmol/l KCl, 25 mmol/l Pipes, 40 mmol/l NaOH, pH 7.2) were added, mixed and incubated for 30 min at room temperature. Then 50 μ l of ¹²⁵J coupled N-acyl histamine and 50 μ l goat anti N-acyl histamine serum²¹, diluted 1:10,000 in PBS²¹ were added and incubated over night at 4°C. During this time, the ¹²⁵J coupled N-acyl histamine formed complexes with the antiserum, depending on the amount of N-acyl histamine in the sample tested. The next day, the addition of 1 ml donkey anti goat serum²² diluted 1:200 in PBS, containing 5% [w/v] PEG 8000, 0.05% [v/v] Triton x 100 and 0.02% [w/v] NaN₃ led to the formation of precipitates (15 min, 4°C), which were harvested by centrifugation (1000 g, 15 min, 4°C). The supernatants were removed completely and the precipitates measured in a Gamma Counter (LKB Wallac 1272 Clinigamma 1272)²³. The histamine concentration was determined using a histamine standard curve ranging from 1–128 ng/ml N-acyl histamine.

Intracutaneous skin reaction

Intracutaneous skin injections were performed with a 26 guage needle in the lateral neck of 4 healthy horses after shaving the injection area. The horses were age 5–14 years, of both sexes and of different breeds. Each injection consisted of a total volume of 100 μ l. The purified NP-IgE was used in concentrations of 3.16, 1 and 0.316 μ g/ml in 0.9% saline (NaCl) as well as 0.9% saline as control. After 18 h, NIP₁₅-BSA (25 μ g/ml in 0.9% saline) was injected into the skin at identical locations as the NP-IgE previously. The reaction was determined by measuring the diameters of the skin swelling and calculation of the respective intracutaneous swelling areas. The intracutaneous reaction was measured after 40–60 min, 2, 5, 8 and 26 h.

Results

Construction of the V_H186.2-C ϵ expression vector encoding a chimeric equi-murine IgE heavy chain

The sequence of the amplified murine V_H186.2 cDNA

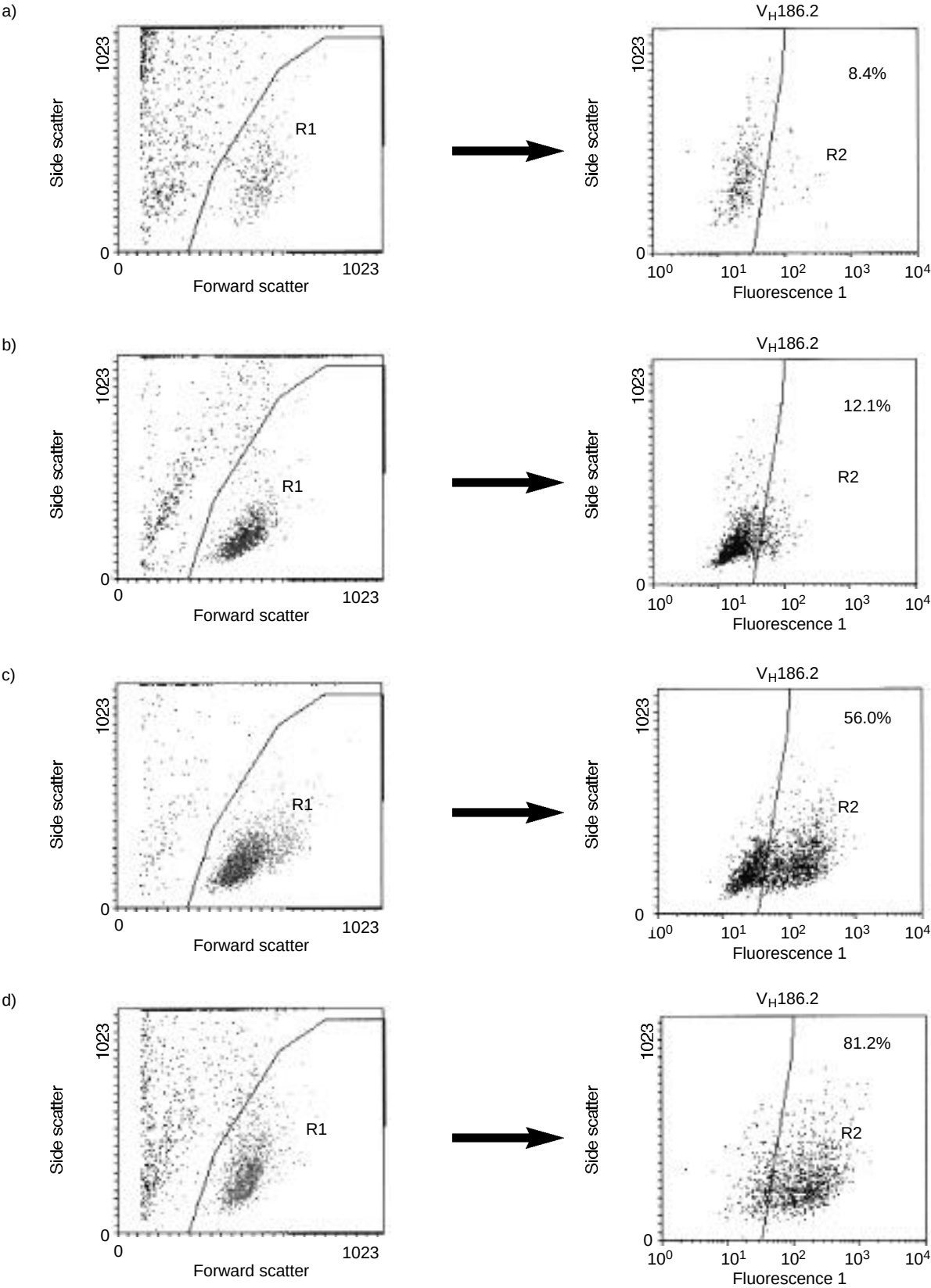


Fig 1: Intracellular staining of the V_H186.2 domain in transfected J558L cells. J558L cells were transfected stably with the V_H186.2-C ϵ expression vector and stained with the monoclonal antibody Ac 146 specific for the V_H186.2 domain. Living cells are shown in the R1 gate. V_H186.2 positive cells (R2) were detected in the population of living cells: a) 24 h after transfection; b) 2 weeks after G418 selection; c) after the first and d) after the second cloning by limiting dilution. After this second limiting dilution cloning step, the stably transfected NP-IgE expressing cell line was designated EqE6.

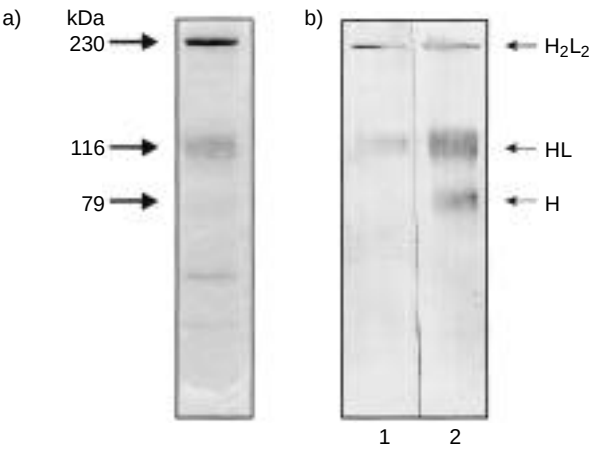


Fig 2: Characterisation of the expressed equi-murine IgE by SDS-PAGE under nonreducing conditions. Purified NP-IgE was separated in a 7.5% SDS-PAGE and (a) stained by Coomassie blue. (b) Equi-murine IgE was identified by Western blotting and detected with either the monoclonal antibody Ac 38 (1), recognising the NP binding site, or an anti-myc antibody (2), detecting the c-terminal myc-epitope of the heavy chain. H₂L₂ = complete equi-murine IgE; HL = dimer, formed by one heavy and one light chain; H = IgE heavy chain.

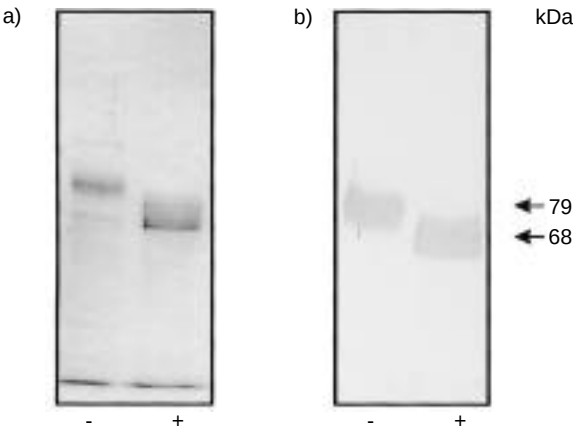


Fig 3: Deglycosylation of the IgE heavy chain by N-glycosidase F treatment. Purified NP-IgE was incubated with and without N-glycosidase F and separated by 10% SDS-PAGE under reducing conditions (+ME). (a) Coomassie blue staining and (b) Western blotting and detection with a myc-specific antibody, detecting the IgE heavy chain.

confirmed that it had 100% homology to the published sequence J00529. As described previously (Wagner et al. 2001), the equine Cε gene contained the complete coding sequence of the Cε^b allele. The 417 bp murine V_H186.2 cDNA and the 1.272 bp equine Cε cDNA were cloned sequentially in the mammalian expression vector. Translation of the coding sequence with 1.773 bp should generate an equi-murine chimeric IgE heavy chain of 591 amino acids. This corresponds to a protein of a predicted molecular weight of 65,600 Da, including the 2,700 Da c-terminal myc-His₆-residue.

Generation of the stably transfected cell line EqE6 expressing NP-specific IgE (NP-IgE)

Murine J558L myeloma cells producing murine λ light chains (Oi et al. 1983) were transfected with the V_H186.2-Cε expression vector. This was expected to lead to the synthesis of

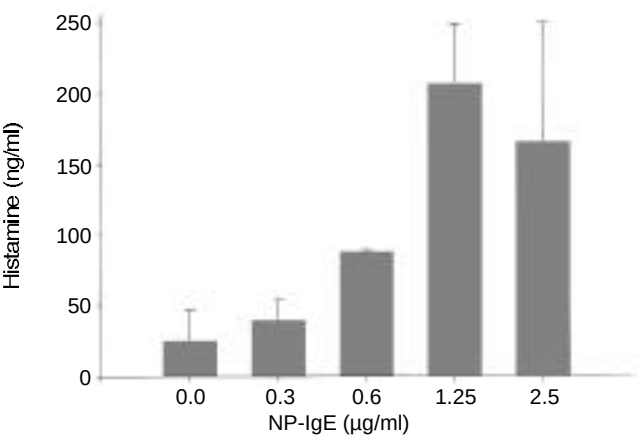


Fig 4: NP-IgE-mediated histamine release in peripheral blood leucocytes after crosslinking with NIP₁₅-BSA. Leucocytes of 2 foals (age 7 days and 2.5 months) were incubated for 2.5 h with different concentrations of NP-IgE, washed and afterwards incubated with NIP₁₅-BSA (25 μg/ml). Histamine was determined in the supernatants by radioimmunoassay. Mean values and s.d. of the histamine amounts released by the 2 foals after crosslinking with NIP₁₅-BSA are shown.

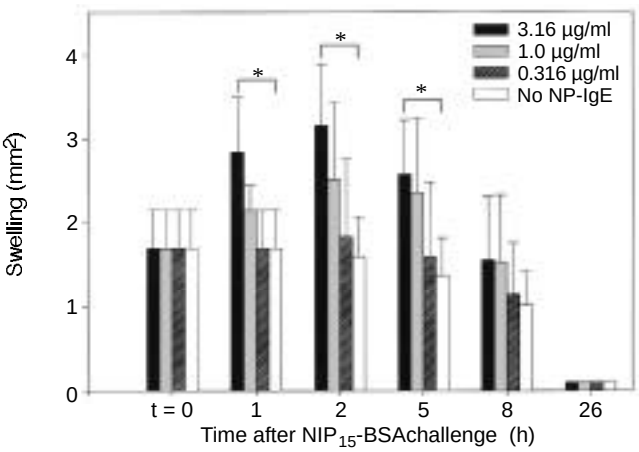


Fig 5: Intracutaneous injection of NP-IgE and crosslinking with NIP₁₅-BSA induced an immediate hypersensitivity reaction. Increasing NP-IgE concentrations were applied in the skin of 4 horses. After 18 h, allowing binding of the NP-IgE to specific mast cell receptors, the antigen NIP₁₅-BSA was challenged at the same skin locations. The skin reaction was then measured over a period of 26 h (*P<0.05). Mean values and s.d. of the swelling of the 4 horses are shown.

immunoglobulins consisting of the light chains originating from the J558L cells and the heavy chains containing the V_H186.2 domain from the transfected vector, yielding antibodies with NP-specific antigen recognition sites. The first test for expression of equi-murine IgE was performed by intracellular staining of the cells and flow cytometric analysis 24 h after transfection, and indicated that 8.4% of the cells expressed the V_H186.2 domain (Fig 1a). For selecting cells that had stably integrated the DNA, transfected cells were seeded in medium containing 1 μg/ml G418 and cell culture supernatants were tested 2 weeks later by ELISA. One NP-IgE-producing culture was identified and cloned twice (Figs 1b–d). After cloning, the NP-IgE cell line, designated EqE6, contained 81.2% of V_H186.2-expressing cells and produced 300–500 ng/ml NP-specific equi-murine IgE.

Biochemical characterisation of the equi-murine IgE

NP-IgE was purified from EqE6 supernatants by affinity chromatography using NP-sepharose columns. The purified protein was characterised by SDS-PAGE and western blotting. A protein of a molecular weight of approximately 230,000 Da was detectable by coomassie blue staining under nonreducing conditions, suggesting that the complete recombinant protein was expressed (Fig 2a). Western blotting was performed using 2 monoclonal antibodies. The first one was specific for the NP antigen binding site formed by both the heavy and the light chain and reacted with the NP-IgE monomer (H_2L_2) and with another band of 116,000 Da, representing a dimer of one heavy and one light chain (HL) (Fig 2b). The second antibody was specific for the c-terminal myc residue, and revealed both bands as well as an additional band of 79,000 Da, corresponding to the single heavy chain.

Under reducing conditions, the heavy chains of 79,000 Da were detected. According to the predicted amino acid sequence, the equine IgE constant heavy chain region should have 6 N-glycosylation sites, 3 in the CH1, one in the CH2 and 2 in the CH3 domain. Indeed, N-glycosidase treatment reduced the molecular weight to 68,000 Da (Fig 3), indicating that the expressed IgE was highly glycosylated. Each heavy chain of the equi-murine IgE included a c-terminal myc-His₆ residue of 2,700 Da. According to the estimated molecular weight of the molecule without this residue, the molecular weight of the unmodified heavy chain should be approximately 76,300 Da and 65,300 Da after deglycosylation.

NP-IgE-mediated histamine release of equine peripheral blood basophils *in vitro* and type I hypersensitivity skin reaction *in vivo*

Peripheral blood leucocytes obtained from 2 foals were incubated with increasing concentrations of NP-IgE to test its binding to free Fc ϵ -receptors, particularly Fc ϵ RI on equine blood basophils. Crosslinking of the Fc ϵ RI via NP-IgE was induced using the specific antigen NIP₁₅-BSA. Histamine concentrations were measured in the supernatants by radioimmunoassay. Increased histamine amounts were released from the foal leucocytes, depending on the NP-IgE concentration used (Fig 4).

The ability of the recombinant NP-IgE to induce mediator release *in vivo* was tested by intracutaneous injection of NP-IgE, followed by NIP₁₅-BSA injection after 18 h (Fig 5). An immediate intracutaneous skin reaction was observed, especially with the highest NP-IgE concentration of 3.16 μ g/ml. Here, the skin reactions were significantly increased ($P < 0.05$) at 1, 2 and 5 h after antigen challenge compared to the control. The skin reaction increased during the first 2 h and had disappeared completely the next day.

These findings suggest strongly that the NP-IgE binds to Fc ϵ RI, most probably on equine basophils and mast cells, and is able to induce the release of mediators from these cells after crosslinking with the antigen *in vitro* and *in vivo*.

Discussion

Certain immunoglobulins of mammalian species have been expressed as recombinant or chimeric immunoglobulins in bacteria or in mammalian cells (Knight *et al.* 1988; Nissim *et al.* 1993; Clarke and Beh 1994). Although protein expression in

mammalian cells results in considerably lower yields than bacterial expression (King *et al.* 1992; Clarke and Beh 1994), for obtaining functional immunoglobulins, particularly of highly glycosylated isotypes like IgE, mammalian expression is more adequate. Immunoglobulin molecules containing complete heavy and light chains can be expressed; and defined antigen recognition sites can be used for detection and purification of the resulting protein. Moreover, proteins expressed in mammalian cells are correctly folded and glycosylated (Shin *et al.* 1992; Clarke and Beh 1994). In contrast, immunoglobulin chains expressed in *E. coli* contain the identical amino acid sequences, but possess lower biological activity, probably due to the loss of the native immunoglobulin domain structure (Henry *et al.* 2000). Whereas the use of eucaryotic expression systems allows the generation and purification of a protein with high similarity to the native IgE, this system is extremely laborious. The positive clones have a comparably low expression level and require constant monitoring of the expression of the recombinant protein.

To obtain a biologically active equine IgE, we constructed the myeloma cell line EqE6, which provides stable expression of chimeric recombinant IgE with a defined specificity for NP. For this, the property of the myeloma cell line J558L to synthesise mouse light chains was combined with the expected ability of the cells to synthesise biologically functional IgE molecules after transfection with the corresponding gene for the equi-murine heavy chain. The secreted protein could be purified readily by affinity chromatography allowing, for the first time, thorough biochemical and biological characterisation of pure equine IgE. Biochemical analysis revealed that the recombinant protein displayed a high structural similarity to the native equine IgE, and maintained specificity for the NP antigen. The NP-IgE was expressed as a tetramer, containing 2 glycosylated heavy chains and 2 light chains.

Comparison of IgE molecules from different species shows that they all possess highly glycosylated constant heavy chain regions with 5 or 6 N-glycosylation sites in the horse, 7 in the human, chimpanzee, pig and dog, 8 in mouse and cattle and 9 in the rat and sheep (Fig 6). Only one site at position N269 of the equine CH3 domain, corresponding to the position N394 in the human IgE, was conserved between all species. Although the differences in the N-glycosylation sites between different species indicate that they are not directly involved in the binding of IgE to its Fc ϵ RI, the one conserved N-glycosylation site was believed to be of major importance (Vernersson *et al.* 1997). However, enzymatically deglycosylated human IgE was still able to interact with the receptor (Helm *et al.* 1988; Sayers *et al.* 1998).

Conversely, glycosylation may have a function in folding and processing of a biologically active IgE molecule. The N-glycosylation site at position N394 in the human IgE is conserved among the human immunoglobulin classes, as well as in all C ϵ 3 domains of mammalian IgE (Fig 6). The N394 site appeared to be essential for the production of a functional IgE molecule by supporting the correct folding of the protein, while mutation of the nonconserved human N371 had no effect on the binding of the IgE to the receptor and on the IgE-mediated antigen-induced cell degranulation (Sayers *et al.* 1998). Therefore, it is conceivable that the conserved N-glycosylation site at N269 in equine IgE that corresponds to N394 in the human IgE influences the folding and formation of a functional IgE binding site in the horse as well as in other mammalian species.

Nothing is yet known regarding the interaction of IgE with

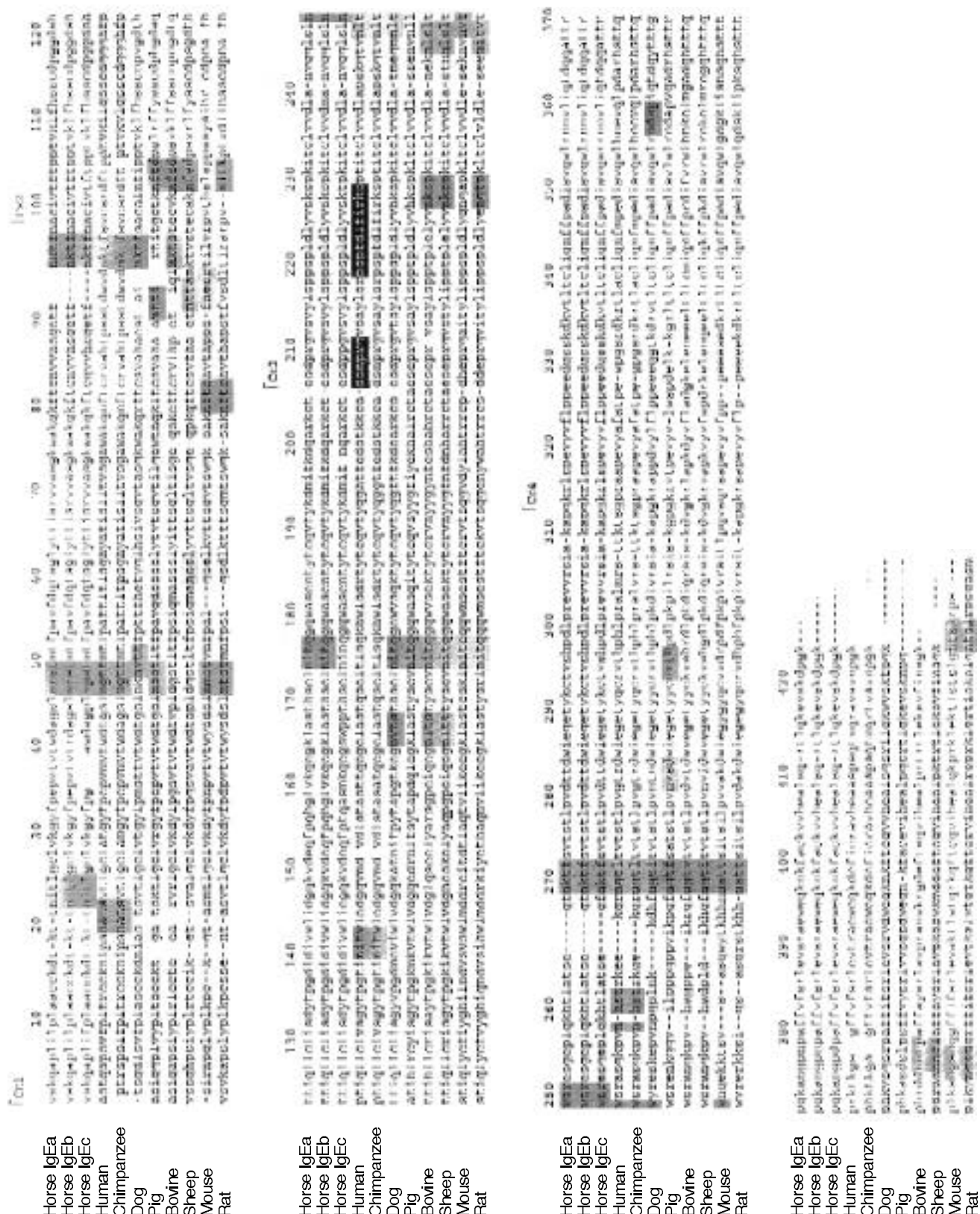


Fig 6: Comparison of the amino acid sequences of the IgE constant heavy chains from different mammalian species. Sequences of the various equine IgE allotypes (IgEc - Navarro et al. 1995 [AA085662]; IgEa and IgEb - Wagner et al. 2001 [A305047, A305048]) were compared with the human (P01854), chimpanzee (AA035416), dog (AA056797), pig (U96100), cattle (AA09546), sheep (AA051378), mouse (EHMS) and rat IgE (EHRT). The potential N-glycosylation sites (N-X-S/T-X) are shaded. The motifs which are expected to be involved in the Fc ϵ RI binding site of the human IgE are marked in black boxes with white letters.

Fc ϵ RI in the horse. Nevertheless, the potential binding sites of human IgE to its Fc ϵ RI have been extensively examined. The C ϵ 2 and C ϵ 3 domains were found to be involved in the binding of IgE to the α chain of the Fc ϵ RI (Helm *et al.* 1988; Nissim and Eshhar 1992). Binding experiments using chimeric IgE molecules have shown that the Fc ϵ RI binding site is predominately located in the C ϵ 3 domain (Nissim *et al.* 1993). Particularly, the N-terminal amino acid residue in the C ϵ 2-3 interface (aa330-335) and the AB loop (aa343-353) in the human C ϵ 3 domain were discussed as the association sites of IgE with the Fc ϵ RI (Sutton and Gould 1993; Sayers and Helm 1999). Interestingly, the human P333 and R334 residues, which were suggested to be involved in the maintenance of conformation required for IgE-mediated effector functions (Sayers *et al.* 1998) and the high affinity interaction of the IgE with the Fc ϵ RI (McDonnell *et al.* 1997) correspond to aa209-210 in the horse, which are conserved in all mammalian species compared in Figure 6. Therefore, equine P209 and R210 could play a comparable role in the interaction of the equine IgE to the high affinity receptor.

However, the entire structure in its native configuration was necessary for the interaction between IgE and the receptor (Nissim *et al.* 1993). The C ϵ 3 domain monomer, expressed in *E. coli*, was able to bind to the Fc ϵ RI, but with a distinctly lower affinity of 5×10^6 /mol/l as compared to 4×10^9 /mol/l for IgE. This may be due either to the loss of the native domain structure, or to the requirement for 2 C ϵ 3 domains (Henry *et al.* 2000). In addition, crystal structure analysis revealed the interaction of the IgE with the Fc ϵ RI on 2 sites, each involving one C ϵ 3 domain of the IgE. This interaction of one receptor with the IgE blocked the binding to a second Fc ϵ receptor α chain (Garman *et al.* 2000). Although the C ϵ 3 domains of the IgE had great influence on the binding on the Fc ϵ RI, the stability of the IgE-Fc ϵ RI complex is a critical factor in allergic responses. Recent studies have shown the stabilising role of the C ϵ 2 domain for the slow dissociation of the IgE-Fc ϵ RI complex, by interaction with both the Fc ϵ RI α chain and the IgE C ϵ 3-C ϵ 4 domains (McDonnell *et al.* 2001).

The ability of the NP-IgE described here to mediate histamine release *in vitro* as well as an immediate skin reaction *in vivo* strongly suggests that the recombinant equi-murine IgE is able to bind to the Fc ϵ RI on equine basophils and mast cells. Therefore, the NP-IgE can be used to investigate the immune mechanisms of IgE-mediated allergic responses, as well as the IgE-Fc ϵ RI interaction, for a better understanding of equine allergic diseases at the cellular level.

In conclusion, the recombinant NP-IgE can be considered to be an extremely useful reagent for the development of monoclonal antibodies specific for equine IgE, which are not yet available, as well as for functional studies of IgE in equine allergic diseases. In addition, the vector can easily be modified for expression of other Ig heavy chains, especially those which cannot be purified from sera or other Ig-rich body fluids, e.g. for the expression of rare IgG isotypes.

Acknowledgements

B1-8 δ and J558L cells, as well as the monoclonal antibodies Ls 136, Ac 38 and Ac 146, were kindly provided by Professor Dr K. Rajewsky, Cologne, Germany. We thank Monica Venner (Clinic of Horses, Hannover, Germany) for the practical performance of the skin test. This work was supported by the Stiftung Gestüt Fährhof and by grants from the Deutsche Forschungsgemeinschaft (Le303/Re426).

Manufacturers' addresses

- ¹Quiagen, Hilden, Germany.
- ²Promega, Mannheim, Germany.
- ³Gibco BRL, Karlsruhe, Germany.
- ⁴Biometra, Göttingen, Germany.
- ⁵Invitrogen, Groningen, The Netherlands.
- ⁶SEQ LAB, Göttingen, Germany.
- ⁷Biochrom, Berlin, Germany.
- ⁸PAN Biotech, Aidenbach, Germany.
- ⁹Cytogen, Lohmar, Germany.
- ¹⁰Bio-Rad Laboratories, München, Germany.
- ¹¹Roth, Karlsruhe, Germany.
- ¹²Sigma, Steinheim, Germany.
- ¹³Dianova, Hamburg, Germany.
- ¹⁴Becton Dickinson, Heidelberg, Germany.
- ¹⁵Nunc, Wiesbaden, Germany.
- ¹⁶Biosearch Technologies, Navato, California, USA.
- ¹⁷Dynatech, Denkendorf, Germany.
- ¹⁸Amersham Pharmacia Biotech, Freiburg, Germany.
- ¹⁹Millipore, Eschborn, Germany.
- ²⁰Roche, Mannheim, Germany.
- ²¹LDN, Nordhorn, Germany.
- ²²Scantibodies Laboratories, Santee, California, USA.
- ²³Turku, Finland.

References

- Bothwell, A.L.M., Paskind, M., Reth, M., Imanishi-Kari, T., Rajewsky, K. and Baltimore, D. (1981) Heavy chain variable region contribution to the b-NP family of antibodies: somatic mutation evident in a gamma-2a variable region. *Cell* **24**, 625-637.
- Clarke, R.A. and Beh, K.J. (1994) Expression of a recombinant sheep IgE gene. *Immunol. Invest.* **23**, 25-37.
- Garman, S.C., Wurzburg, B.A., Tarchevskaya, S.S., Kinet, J.P. and Jardetzky, T.S. (2000) Structure of the Fc fragment of the human IgE bound to its high-affinity receptor Fc epsilonRI alpha. *Nature* **406**, 259-266.
- Helm, B., Marsh, P., Vercelli, D., Padlan, E., Gould, H. and Geha, R. (1988) The mast cell binding site on human immunoglobulin E. *Nature* **331**, 180-183.
- Henry, A.J., McDonnell, J.M., Ghirlando, R., Sutton, B.J. and Gould, H.J. (2000) Conformation of the isolated Cepsilon3 domain of IgE and its complex with the high-affinity receptor, FcepsilonRI. *Biochem. J.* **39**, 7406-7413.
- King, D.J., Adair, J.R., Angal, S., Low, D.C., Proudfoot, K.A., Lloyd, J.C., Bodmer, M.W. and Yarranton, G.T. (1992) Expression, purification and characterization of a mouse-human chimeric antibody and chimeric Fab' fragment. *Biochem. J.* **281**, 317-323.
- Knight, K.L., Suter, M. and Becker, R.S. (1988) Genetic engineering of bovine Ig. Construction and characterization of hapten-binding bovine/murine chimeric IgE, IgA, IgG1, IgG2 and IgG3 molecules. *J. Immunol.* **140**, 3654-3659.
- Kozak, M. (1989) The scanning model for translation: an update. *J. cell Biol.* **108**, 229-241.
- Larsen, H.J., Bakke, S.H. and Mehl, R. (1988) Intradermal challenge of Icelandic horses in Norway and Iceland with extracts of *Culicoides* spp. *Acta vet. Scand.* **29**, 311-314.
- Mansfield, J.R., Burrell, M.H., Curl, V.J. and Valler, B.J. (1998) Treatment of equine allergic diseases with allergy neutralization. A field study. *J. Nutr. Environ. Med.* **8**, 329-334.
- Marti, E., Peveri, P., Griot-Wenk, M., Muntwyler, J., Crameri, R., Schaller, J., Gerber, H. and Lazary, S. (1997) Chicken antibodies to a recombinant fragment of the equine immunoglobulin epsilon heavy-chain recognising native horse IgE. *Vet. Immunol. Immunopath.* **59**, 253-270.
- Matthews, A.G., Imlah, P. and McPherson, E.A. (1983) A reagin-like antibody in horse serum: 1. Occurrence and some biological properties. *Vet. res. Commun.* **6**, 13-23.
- McDonnell, J.M., Beavil, A.J., Mackay, G.A., Henry, A.J., Cook, J.P.D., Gould, H.J. and Sutton, B.J. (1997) Structure-based design of peptide that inhibit IgE binding to its high affinity receptor Fc ϵ RI. *Biochem. Soc. Trans.* **25**, 387-392.
- McDonnell, J.M., Calvert, R., Beavil, R.L., Beavil, A.J., Henry, A.J., Sutton B.J., Gould, H.J. and Cowburn, D. (2001) The structure of the IgE C ϵ 2 domain and its role in stabilizing the complex with its high-affinity receptor Fc ϵ 2RIalpha. *Nat. Struct. Biol.* **8**, 437-441.
- McGorum, B.C., Dixon, P.M. and Halliwell, R.E.W. (1993) Quantification of histamine in plasma and pulmonary fluids from horses with chronic obstructive pulmonary disease, before and after 'natural (hay and straw) challenges'. *Vet. Immunol. Immunopath.* **36**, 223-237.

- Navarro, P., Barbis, D.P., Antczak, D. and Butler, J.E. (1995) The complete cDNA and deduced amino acid sequence of equine IgE. *Mol. Immunol.* **32**, 1-8.
- Neuberger, M.S. and Rajewsky, K. (1981) Switch from hapten-specific immunoglobulin M to immunoglobulin D secretion in a hybrid mouse cell line. *Proc. natl. Acad. Sci. USA.* **78**, 1138-1142.
- Nissim, A. and Eshhar, Z. (1992) The human mast cell receptor binding site maps to the third constant domain of immunoglobulin E. *Mol. Immunol.* **29**, 1065-1072.
- Nissim, A., Schwarzbaum, S., Siraganian, R. and Eshhar, Z. (1993) Fine specificity of the IgE interaction with the low and high affinity Fc receptor. *J. Immunol.* **150**, 1365-1374.
- Oi, V.T., Morrison, S.L., Herzenberg, L.A. and Berg, P. (1983) Immunoglobulin gene expression in transformed lymphoid cells. *Proc. natl. Acad. Sci. USA* **80**, 825-829.
- Reth, M., Imanishi-Kari, T. and Rajewsky, K. (1979) Analysis of the repertoire of anti-(4-hydroxy-3-nitro-phenyl)acetyl (NP) antibodies in C57BL/6 mice by cell fusion. II. Characterization of idiotypes by monoclonal anti-idiotypic antibodies. *Eur. J. Immunol.* **9**, 1004-1013.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York.
- Sayers, I., Cain, S.A., Swan, J.R.M., Pickett, M.A., Watt, P.J., Holgate, S.T., Padlan, E.A., Schuck, P. and Helm, B.A. (1998) Amino acid residues that influence FcεRI-mediated effector functions of human immunoglobulin E. *Biochem.* **37**, 16152-16164.
- Sayers, I. and Helm, B.A. (1999) The structural basis of human IgE-Fc receptor interactions. *Clin. exp. Allergy* **29**, 585-594.
- Schmallenbach, K.H., Rahman, I., Sasse, H.H.L., Dixon, P.M., Halliwell, R.E.W., McGorum, B.C., Crameri, R. and Miller, H.R.P. (1998) Studies on pulmonary and systemic *Aspergillus fumigatus*-specific IgE and IgG antibodies in horses affected with chronic obstructive pulmonary disease (COPD). *Vet. Immunol. Immunopath.* **66**, 245-256.
- Shin, S.-U., Wei, C.F., Amin, A.R., Thorbecke, J. and Morrison, S.L. (1992) Structural and functional properties of mouse-human chimeric IgD. *Hum. Antibodies Hybridomas* **3**, 65-74.
- Suter, M. and Fey, H. (1981) Isolation and characterization of horse IgE. *Zbl. vet. Med. B* **28**, 414-420.
- Sutton, B.J. and Gould, H.J. (1993) The human IgE network. *Nature* **366**, 421-428.
- Ungar-Waron, H., Braverman, Y., Gluckman, A. and Trainin, Z. (1990) Immunogenicity and allergenicity of *Culicoides imicola* (Diptera: Ceratopogonidae) extracts. *J. vet. Med. B* **37**, 64-72.
- Vernersson, M., Pejler, G., Kristersson, T., Alving, K. and Hellmann, L. (1997) Cloning, structural analysis, and expression of the pig IgE ε chain. *Immunogenet.* **46**, 461-468.
- Wagner, B., Radbruch, A., Richards, C. and Leibold, W. (1995) Monoclonal equine IgM and IgG immunoglobulins. *Vet. Immunol. Immunopath.* **47**, 1-12.
- Wagner, B., Siebenkotten, G., Radbruch, A. and Leibold, W. (2001) Nucleotide sequence and restriction fragment length polymorphism of equine Cε gene. *Vet Immunol. Immunopath.* **82**, 193-202.
- Watson, J.L., Pettigrew, H.D., Wilson, L.K. and Gershwin, L.J. (1997) Isolation and partial expression of a cDNA clone encoding equine IgE heavy chain and production of polyclonal antibodies to the recombinant protein. *J. vet. Allergy clin. Immunol.* **5**, 135-142.

Received for publication: 6.8.01

Accepted: 17.10.01