indicates that $T_4$ was at a lower level in older patients. Thyroxine levels are regulated by thyroid stimulating hormone secreted by the pituitary anterior lobe which in turn is regulated by thyrotropin releasing hormone secreted from the hypothalamus[18]. Factors regulating the secretion of TRH are uncertain but may include influences from higher centres and a stimulatory effect of the thyroid hormones[18] presumably acting through receptors. Dystrophin is known to be associated with normal brain cells[19]. An abnormality in brain cells in DMD patients may affect functioning of the hypothalamus and hence the hypothalamus-pituitary-thyroid axis in different ways at early and later stages of the disease.

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## Immunological comparison between albumins of three species of mice (genus *Mus*)

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*Summary.* Three closely related species of short-tailed mice (*Mus musculus musculus*, *M. spretoides* and *M. spicilegus*) were tentatively discriminated using immunological techniques based on albumin cross-reactivity. Different fractionations of crude albumin antisera allowed the recovery of antibody populations specific to the *M. m. musculus* albumin, whereas antibody population differences do not seem to exist between *M. spicilegus* and *M. spretoides*. Moreover, immunoreactivities tested with native and S-carboxymethylated albumins revealed that species-specific antibodies correspond to antigenic determinants depending on the amino acid sequence (sequential determinants). The observed immunological differences are related to species divergence and albumin sequences.
*Key words. Mus musculus musculus*; *Mus spicilegus*; *Mus spretoides*; albumin antiserum fractionation; native and S-carboxymethylated albumins.

The use of biochemical techniques, particularly protein electrophoresis, has made it possible to redefine the systematics of the genus *Mus*[1, 2] and to ascribe an accurate taxonomic rank to species which are very similar morphologically. Four biochemical groups of mice were thus recognized in Central and Eastern Europe[3], corresponding to *Mus musculus domesticus*, *M. m. musculus*, *M. spicilegus* and *M. spretoides* (although the nomenclature of the latter taxa was still debated, see Auffray et al.[4]). Among these, *M. m. domesticus* is the only long-tailed mouse. The three remaining taxa are all relatively short-tailed and are thus very difficult to differentiate in the field.

Albumin has often been used as an evolutionary marker, and in immunological comparisons aimed at quantifying the degree of divergence of the taxa considered[5, 6]. In the

present study, we used immunological techniques to compare qualitatively albumins of the three European taxa of short-tailed mice, in order to find a specific marker making it possible to differentiate the three species. The discrimination is based on various fractionations of albumin antisera and is performed using western-blotting and the enzyme-linked immunosorbent assay (ELISA) method. Furthermore, the nature of antigenic determinants involved in the differentiation between species was investigated using the reactivity of fractionated antisera with native and denatured albumins. The observed immunological differences are finally discussed in relation to albumin sequences and compared with the results given by protein electrophoresis.

*Materials and methods*

*Albumin purification and production of antisera.* The three species *M. m. musculus*, *M. spretoides* and *M. spicilegus* used for the immunological analysis are maintained as wild-derived strains and were kindly provided by Dr F. Bonhomme (Montpellier). The albumin of each species was prepurified from whole serum by precipitation with ammonium sulfate[5]. The prepurified albumin was then passed through an Affy-Blue column (Cibacron-Blue Sepharose) equilibrated with 0.05 M-Tris/HCl/0.05 M-NaCl pH 8.0 and the fixed albumin recovered by desorption with 0.05 M-Tris/HCl/0.2 M-NaSCN pH 8.0, according to the method of Travis et al.[7]. Albumin purity was verified by SDS-polyacrylamide electrophoresis[5]. Antibodies to each albumin species were obtained using New Zealand white rabbits following the immunization procedure previously described[5].

*Serum fractionation.* Purified albumin was linked to Sepharose 4B (Pharmacia) by the CNBr procedure[8] and used to prepare columns. Antiserum was then passed through the appropriate insolubilized albumin equilibrated with PBS (0.14 M-NaCl/0.01 M sodium phosphate buffer pH 7.4). The flow-through fraction was recovered and stored at $-5\,^{\circ}C$ in 50% glycerol. The retained antibodies were eluted in 10% dioxan/20 mM-$Na_3PO_4$ pH 12.0[9], immediately neutralized at 4 °C with crystals of $NaH_2PO_4$ and dialyzed against PBS.

*S-carboxymethylation.* Albumin was denatured in 6 M-guanidine chloride/0.1 M-sodium phosphate, pH 8.8 and reduction was performed at 37 °C overnight using dithiothreitol (50 moles/mole of disulfide in the protein)[10]. Then, 0.1 M iodoacetic acid was added and allowed to react for 30 min in the dark. The reagents were removed by dialysis against sodium hydrogen phosphate (0.1 M, pH 9). Complete S-carboxymethylation of albumins was checked by amino acid analysis after 24 h hydrolysis in 6 N HCl at 110 °C.

*Western-blotting.* After electrophoresis on a SDS-acrylamide slab gel, proteins were electrotransferred to a nitrocellulose filter[11]. Free sites of the filter were saturated by a 1-h incubation in a PBS solution of defatted powdered milk at 80 g/l. After washing twice in PBS for 10 min, the filter was incubated for 1 h at 37 °C with antibodies. The nitrocellulose was then rinsed four times with PBS containing 0.2% Tween 20 and resaturated in the milk solution for 30 min. After two washings in PBS, the antigenic reactivity was detected by incubation at 37 °C for 1 h in goat anti-rabbit immunoglobulins conjugated with horseradish peroxidase (Biosys, France) diluted 1/500 in PBS. The peroxidase activity was demonstrated using 4-chloro-1-naphthol in 0.01 M-$H_2O_2$/0.05 M-Tris/HCl pH 7.6. The reaction was stopped when color development was sufficient, by rinsing the filter several times in distilled water.

*ELISA.* The test[12] was carried out in microtiter plates as previously reported[5]. Two methods, direct and competitive ELISA, were used. In the direct method, wells were coated with native or S-carboxymethylated albumins of the three species and the first antibody added in serial dilutions. In the competitive ELISA, wells were coated with albumin of *M. m. musculus*, and free antigen (native albumins of *M. m. musculus*, *M. spicilegus* and *M. spretoides*) was added in increasing concentrations together with the first antibody, the dilution of which was constant.

*Results*

*Reactivity of whole antisera.* Rabbit antisera produced against the albumins of *M. m. musculus*, *M. spicilegus* and *M. spretoides* were tested by western-blotting against the serum (used as antigen) of the three mice species. Each of the antisera is specific for albumin but recognizes the three species (fig. 1, A and B). When tested by direct ELISA, all antisera showed almost identical reactivities with the three albumins. Only one antiserum directed against *M. m. musculus* revealed a slight difference between the homologous species and the two others (fig. 2).

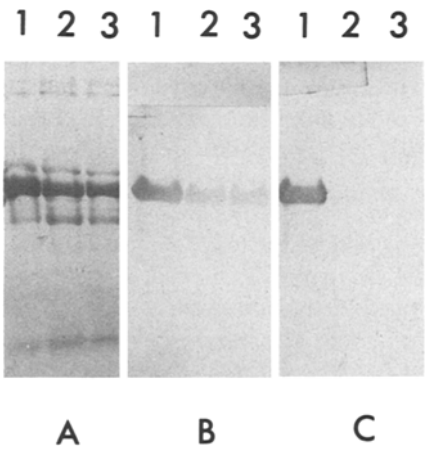


Figure 1. Electrophoretic blotting with *M. m. musculus* (1), *M. spicilegus* (2) and *M. spretoides* (3). *A* Coomassie blue stained polyacrylamide gel after electrophoresis of crude serum. *B* and *C* Nitrocellulose filter replicate revealed with whole *M. m. musculus* albumin antiserum (*B*) and with the fraction of this same antiserum non retained on an *M. spretoides* column (*C*).

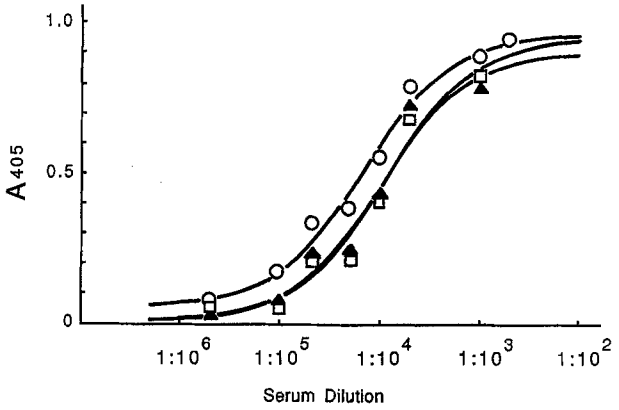


Figure 2. Reactivity of whole *M. m. musculus* antiserum with the native albumins of *M. m. musculus* (○), *M. spicilegus* (▲) and *M. spretoides* (□) tested by direct ELISA.

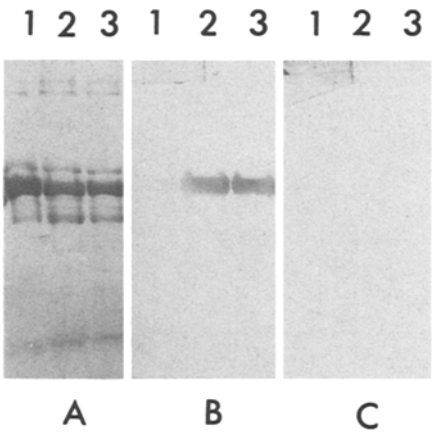


Figure 3. Electrophoretic blotting with *M. m. musculus* (1), *M. spicilegus* (2) and *M. spretoides* (3). *A* Coomassie blue stained polyacrylamide gel after electrophoresis of crude serum. *B* and *C* Nitrocellulose filter replicate revealed with antiserum *M. spicilegus* non-retained on *M. m. musculus* (*B*) and on *M. m. musculus* and *M. spretoides* (*C*).

Thus it can be concluded that antigenic sites common to the three species are numerous enough for the various albumins to be equally recognized by the crude antisera.

*Reactivity of fractionated antisera.* The three species were then tentatively discriminated using serum fractionation on insolubilized albumin columns.

In a first experiment, *M. m. musculus* antiserum was passed through an *M. spretoides* albumin column which retained the antibodies that recognize *M. spretoides*, i.e. the antibodies common to *M. m. musculus* and *M. spretoides*. As shown in figure 1 A and 1 C, the non-retained fraction tested against the three species reacted only with *M. m. musculus*. The same results were obtained when the serum was fractionated on an *M. spicilegus* albumin column. In a second test, *M. spretoides* antiserum was first fractionated on an *M. m. musculus* column and half of the non-retained fraction was passed through insolubilized *M. spicilegus* albumin. The test of the two non-retained fractions is presented in figure 3. The first fraction did not react with *M. m. musculus* but recognized both *M. spretoides* and *M. spicilegus* (fig. 3, A and B). On the other hand, the fraction that was not retained on *M. m. musculus* and *M. spicilegus* did not respond to the three albumins (fig. 3, A and C). The latter test involved the fraction of the *M. spicilegus* antiserum that is not retained on an *M. spretoides* column. This fraction did not react at all with any of the albumins tested (data not shown).

When tested by competitive ELISA (fig. 4), the antiserum *M. m. musculus* fractionated on *M. spretoides* confirmed that these antibodies react neither with *M. spretoides* nor with *M. spicilegus*. As a matter of fact, these two albumins cannot prevent the fixation of antibodies on the adsorbed *M. m. musculus* albumin.

*Reactivity with S-carboxymethylated albumins.* The percentage amino acid content of native and S-carboxymethylated albumins of *M. m. musculus* (table) did not differ much from those of human, rat and bovine albumin given by Peters [13] (see rat albumin content in the

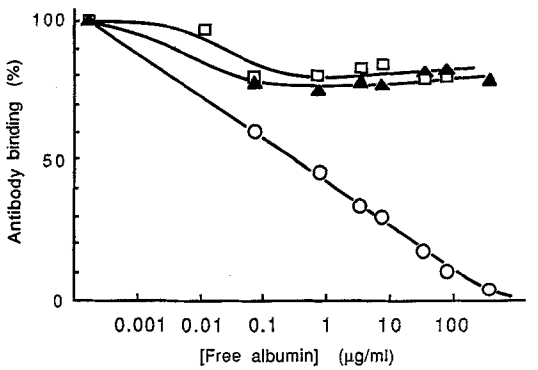


Figure 4. Competitive ELISA performed with *M. m. musculus* antiserum fractionated on *M. spretoides*. Wells were coated with native *M. m. musculus* albumin and antiserum (dilution 1/50) was added with increasing concentrations of free native albumins of *M. m. musculus* (○), *M. spicilegus* (▲) and *M. spretoides* (□). The percent of antibody binding was determined, taking the maximal absorbance in absence of free antigen as 100%.

Percent amino acid composition of native and carboxymethylated albumins of *M. m. musculus* and rat albumin

| Amino acid | *M. m. musculus* native | *M. m. musculus* carboxymethylated | Rat[a] |
|---|---|---|---|
| CMC[b] | – | 6.70 | – |
| Asx | 9.68 | 9.37 | 8.90 |
| Thr | 7.02 | 6.75 | 5.65 |
| Ser | 5.02 | 5.00 | 4.11 |
| Glx | 14.95 | 14.38 | 14.04 |
| Pro | 5.14 | 4.76 | 5.14 |
| Gly | 3.08 | 3.23 | 2.91 |
| Ala | 9.98 | 9.59 | 10.44 |
| Val | 5.46 | 5.20 | 5.99 |
| Cys | – | – | 5.99 |
| Met | 1.34 | 1.00 | 1.02 |
| Ile | 1.63 | 1.58 | 2.23 |
| Leu | 10.88 | 10.28 | 9.59 |
| Tyr | 3.58 | 3.51 | 3.59 |
| Phe | 4.31 | 4.10 | 4.45 |
| Trp | – | – | 0.17 |
| Lys | 8.74 | 8.16 | 9.07 |
| His | 2.88 | 2.70 | 2.57 |
| Arg | 3.85 | 3.67 | 4.11 |

[a] Calculated from the sequence given by Peters [13]. [b] Carboxymethyl-cysteine.

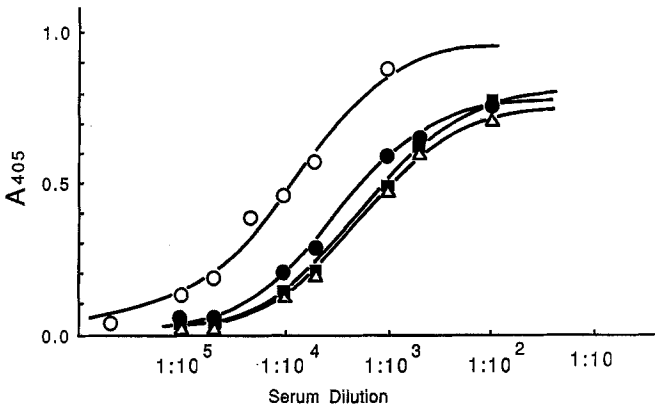


Figure 5. Immunoreactivity of whole *M. m. musculus* antiserum tested by direct ELISA against the native albumin of *M. m. musculus* (○) and the S-carboxymethylated albumins of *M. m. musculus* (●), *M. spicilegus* (△) and *M. spretoides* (■).

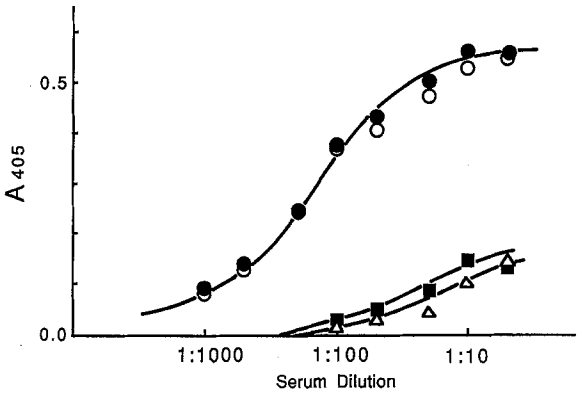


Figure 6. A Coomassie blue polyacrylamide gel after electrophoresis of native and S-carboxymethylated albumins of *M. m. musculus* (1 and 4), *M. spicilegus* (2 and 5) and *M. spretoides* (3 and 6). *B* and *C* Nitrocellulose filter after blotting of denaturated albumins revealed with antiserum *M. m. musculus* non-retained on *M. spretoides* (*B*) and with antiserum *M. spretoides* non-retained on *M. m. musculus* (*C*).

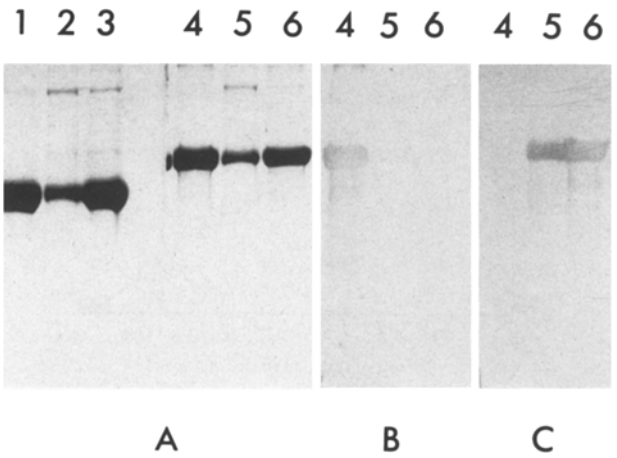


Figure 7. Immunoreactivity of *M. m. musculus* antiserum fractionated on *M. spretoides* tested by direct ELISA against the native albumin of *M. m. musculus* (○) and the S-carboxymethylated albumins of *M. m. musculus* (●), *M. spicilegus* (△) and *M. spretoides* (■).

table). In particular, the percentage of cysteine residues was similar, indicating that albumin was totally S-carboxymethylated. Complete cleavage of disulfide bonds was also obtained for the albumins of the two other species. The immunoreactivity of the whole *M. m. musculus* antiserum against the three reduced albumins and the native form of *M. m. musculus* was tested by direct ELISA (fig. 5). It can be seen that the denatured albumins do not differ between them but the curves show a shift both in reactivity and in the plateau level as compared with the native albumin.

We then used native and denatured albumins to check the nature (sequential vs conformational, see discussion) of the antibodies remaining in two fractionated antisera. When tested by immunoblotting against the carboxymethylated albumins, the antiserum specific to *M. m. musculus* reacted only with the denaturated albumin of *M. m. musculus* (fig. 6, A and B). Similarly, the fractionated antiserum which recognizes the *M. spicilegus* and *M. spretoides* albumins also responded to the denaturated forms (fig. 6, A and C). With both antisera, the intensity of the reactions is comparable to that of the native albumins. The fractionated antiserum specific to *M. m. musculus* was also tested by direct ELISA with the three S-carboxymethylated albumins *plus* the native albumin of *M. m. musculus*. As shown in figure 7, very weak reactions occurred with the two heterologous denaturated albumins whereas the reactivity was the same for S-carboxymethylated and native albumins of *M. m. musculus*. Therefore fractionated antisera recognize the S-carboxymethylated as well as the native forms of homologous albumins.

## *Discussion*

*Obtaining an immunological marker.* The results obtained with serum fractionations revealed that, when compared with *M. spretoides* and *M. spicilegus*, there are antibody populations of *M. m. musculus* antiserum that do not recognize these two species, and therefore are specific to the *M. m. musculus* albumin. By contrast, antibody population differences do not seem to exist between the *M. spretoides* and *M. spicilegus* antisera, since all the *M. spretoides* (or *M. spicilegus*) albumin antibodies are retained on an *M. spicilegus* (or *M. spretoides*) albumin column. We thus have a fractionated antiserum which allows the discrimination of *M. m. musculus* from the two other species. Used in the field, this immunological marker will be useful for the specific identification of living *M. m. musculus* individuals among a sample of wild-caught mice (Montgelard et al., in preparation).

*Nature of antigenic determinants.* The antibodies involved in the differentiation between these species have been dealt with in a comparative study of native and S-carboxymethylated albumins. As a matter of fact, antibodies are usually thought to fall into two categories of

antigenic determinants. Although all determinants are now seen to be topographic[14], they are classified either as conformational or discontinuous determinants depending on the native spatial conformation of the protein, or as sequential or continuous determinants depending rather on the amino acid sequence. Since the reductive cleavage of disulfide bonds followed by S-carboxymethylation in 6 M guanidine results in an unfolded protein[15], antibodies which still recognize the denaturated form are assumed to belong to the sequential class of determinants. The results obtained with crude antisera reveal a clear shift in immunoreactivity of S-carboxymethylated albumins with respect to the native form (fig. 5). This phenomenon has been known for some time[15]: denaturation is associated with a decrease in antigenicity (with antibodies directed against the native protein) which is related to the degree of unfolding of the polypeptide chain.

In contrast, fractionated antisera show no difference in reactivity between native and S-carboxymethylated homologous albumins whereas heterologous albumins do not react (fig. 7). Therefore, we can conclude that fractionated antisera specific either to *M. m. musculus* or to *M. spicilegus* and *M. spretoides* contain antibodies which correspond mostly to sequential antigenic determinants; that is, they are mainly directed against the parts of the sequences showing species differences. Moreover, it is clear that disulfide bonds are not involved in the production of species-specific antibodies. There are two explanations for this result. Firstly, -S-S- bridges participate in the tertiary structure of the molecule, which is a feature common to all mammalian albumins known so far[13], and hence probably also occurs in the rabbit which is the antibody-producing species. Secondly, it is known that disulfide bonds per se do not play a major role in determining antigenicity since they merely stabilize the thermodynamically most stable conformation[15].

*Species divergence and albumin sequences.* The absence of immunological differences between *M. spicilegus* and *M. spretoides* confirms that these two species are very close, as has already been shown with electrophoretic markers. According to Bonhome et al.[16], there are only two diagnostic loci between these two species (*Alb*-1 and *Es*-3), and the Nei genetic distance index is 0.07 (for 42 loci), which is a very low value between two species. As a comparison, the corresponding genetic distance between the two subspecies *M. m. musculus* and *M. m. domesticus* amounts to 0.35 for the same loci.

Moreover, our results provide some information concerning albumin sequences, especially with respect to alleles of the *Alb*-1 locus scored by electrophoresis. The various electromorphs are distributed as follows[16]: *M. spretoides* carries *Alb*-1$^{c}$, whereas *M. m. musculus* and *M. spicilegus* share *Alb*-1$^{a}$ (our electrophoresis patterns do not show such electromorphs because they are performed in the presence of SDS). By contrast, our immunological method, which cannot discriminate between *M. spretoides* and *M. spicilegus*, differentiates *M. m. musculus* from the two other species. These results indicate that although *Alb-1*$^{c}$ of *M. spretoides* and *Alb-1*$^{a}$ of *M. spicilegus* are electrophoretically distinct, the number of different antigenic sites must be very low, and therefore the two albumin amino acid sequences probably have very few differences. On the other hand, our results reveal that the same *Alb-1*$^{a}$ electromorph in *M. m. musculus* and *M. spicilegus* corresponds in fact to two divergent molecules, probably showing an important divergence in their sequences.

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