Préhistoire Européenne, volume 11, 1997, p. 331 à 336.

TAXONOMIC SPECIFICITY OF FOSSIL COLLAGEN MOLECULES IN ENZYME LINKED IMMUNO ASSAY

P. SEMAL*

1. INTRODUCTION

Bone and teeth are the best preserved tissue of animal bodies during fossilisation. The organic fraction represents about 30% of the fresh bone weight. Collagen is the most abundant protein in bone tissue and represents up to 90% of the bone proteins. Chemical links with hydroxyapatite molecules make collagen a well-preserved protein during some fossilisation processes. Collagen is used for ^{14}C dating but other applications like palaeoimmunology on the organic fraction can also provide information.

ABO blood group determination (palaeoserology) was the first application of immunological techniques on fossil human remains (CANDELA, 1936).

Antibodies can also be used in taxonomic determination of bone fragments (LOWENSTEIN, 1980; SHOSHANI *et al.*, 1985, SEMAL, 1996).

More recently, antibodies were used in blood residues analyses on archaeological tools (KOOYMANN *et al.*, 1992) and in palaeopathological diagnostics (CATTANEO *et. al.*, 1995).

The central problem of paleoimmunology is represented by the antibody/antigen specificity which is submitted to degradation and contamination of the antigens. ABO blood group analysis is then restricted to specimens younger than a few thousands years. Many antigens like haemoglobin (ASCENZI *et al.*, 1985, SMITH and WILSON, 1990) or immunoglobulins (CATTANEO *et al.*, 1992) present the same limits in time as ABO blood groups.

Other bone proteins like osteocalcin, albumin and collagen are quite well preserved during fossilisation. Osteocalcin is the best preserved protein (several million years), but it presents poor taxonomic specificity (ULRICH *et al.*, 1987). Albumin was extensively used as an antigen in comparative taxonomy on modern specimens (GOODMAN, 1962). It is a good antigen for such analyses with fossils remains (LOWENSTEIN, 1981), but the amount of albumin in bone is low compared to osteocalcin and collagen thus necessitating the destruction of a larger amount of fossil bone to obtain adequate amounts of albumin.

Like for palaeoserology, the central problem in immunological taxonomic analyses of fossil material is the degradation of the antigenic sites during fossilisation. Previous studies (SHOSHANI *et al.*, 1985) show that fossils antigens are in part destroyed and thus less recognised by antibodies than recent control samples.

The aim of this study, supported by the SC-004 program, was the determination of antigenic properties of collagen in the taxonomic diagnosis of fossil bones (SEMAL, 1996). Indeed, specific diagnosis of fragmentary human bone is important for studies of human remains while differentiation of sister species (e.g., *Rangifer tarandus/Cervus Elaphus* and *Bos primigenius/Bison priscus*) is useful for

* Royal Belgian Institute of Natural Sciences, Vautierstreet 29, B-1000 Brussels.

climate reconstruction. Moreover, bone artefacts loose the morphological features which allow their taxonomic diagnose; better taxonomic identification of animal bone could help archaeologists in determining the most frequently used bones in prehistoric artefact construction. Therefore, we test here the possibility of diagnosing collagen molecules obtained from antlers or bones of *Cervidae*.

2. EXTRACTION AND QUANTITATIVE ANALYSIS OF COLLAGEN

We developed a collagen extraction technique designed for recent and fossil bones (SEMAL and ORBAN, 1995). This method extracts large amount of collagen peptides (> 10 kDa) from small bone samples (< 100 mg). The quantification of hydroxyproline, a particular amino acid of collagen, allows us to specifically measure the collagen in our bone extracts, as an independent quantification technique of the antigen is needed for the interpretation of the immunological results.

We have tried to use this technique as a preparative procedure for radiocarbon dating but the age obtained was 10% younger than expected. This method which does not use NaOH treatment probably leaves humic contaminants in the protein extract.

3. IMMUNOLOGICAL RESULTS

Rabbits and hens were immunised with bone extracted collagen. Antibodies against human collagen, cervid collagen (deer, reindeer, elk, fallow deer and *Megaloceros*), bovid collagen molecules (cow and bison) and probocid collagen (mammoth and elephant) were then purified from the rabbit blood and from the hens' egg yolk (SEMAL, 1996).

Immunological reactions were tested by ELISA (Enzyme Linked Immuno Assay) with a direct coating of the antigens on the polyvinyl plate or by competition in a liquid phase (Semal, 1996).

3.1. Taxonomical specificity of the antibodies obtained tested with recent collagen

We obtained 3 different specificity levels :

- No taxonomic specificity inside of the mammals : Anti-red deer collagen antibodies possess no specificity and recognises all mammal collagen tested. They probably fix the central amino acid sequences of collagen which are very similar throughout the vertebrates.

- Family specificity : Anti-fallow deer collagen, elk collagen and reindeer antibodies differentiate cervid collagen from the collagen of other mammals (human, primate or elephant).

- *Intra*-family specificity : Anti-reindeer collagen antibodies possess the best specificity and are able to differentiate between the collagen of different cervid species.

In the following experiment, different diluted solutions of anti-reindeer collagen antibodies were tested on collagen from reindeer, elephant and human collagen (500 ng / well), and milk proteins. The antibodies are only fixed on the reindeer collagen (Figure 1).

3.2. Taxonomical specificity of antibodies tested with recent and fossil collagen

We also tested the specificity of these anti-reindeer collagen antibodies (diluted 160 X) for extracts from cervid antlers (recent and fossils) and human bones (recent and fossil) (2000 to 31 ng / well) (Figure 2). The antibodies are fixed only by the recent reindeer collagen while the fossil reindeer and *Megaloceros* collagen are not recognised. Fossil homologous collagen molecules are poorly recognised in this direct fixation test.

Nevertheless, in competition, a fraction of antigenic sites from fossil reindeer collagen inhibits significantly the fixation of anti-reindeer collagen antibodies while human collagen (recent and fossil) and *Megaloceros* (fossil) do not, thus allowing taxonomic diagnosis (Figure 3).

3.3. Taxonomical specificity of antibodies obtained using fossil collagen

We also tested the capacity of fossil proteins to stimulate the hen's immune system in order to obtain some specific antibodies. We immunised hens with mammoth or *Megaloceros* collagen. Antibodies were tested in direct and competition ELISA.

Anti-mammoth collagen antibodies distinguish between elephantid collagen and other mammals collagens (Figure 4). Mammoth collagen is also well recognized by anti-African elephant collagen antibodies. Fossil proteins could then be used to produce antibodies with taxonomical specificity. However more than 15 mg of collagen are needed for one immunisation procedure while only a few micrograms are sufficient to test the antigenic capacities of fossil collagen with antibodies raised against recent collagen samples.

Finally we have tested the specificity of anti-*Megaloceros* collagen antibodies (not showed). Collagen of cervids (reindeer and *Megaloceros*) in liquid phase (1000-62 ng / 50 µl) inhibits the fixation of antibodies (diluted 50X) on the *Megaloceros* collagen solid phase (500 ng / well), while elephant and mammoth collagen in liquid phase do not. However human collagen in liquid phase inhibits this fixation and shows that spurious cross-reactions can be obtained while general taxonomic specificity seems to be good.

3.4. Taxonomical specificity of antibodies tested with collagen extracted from hearth bones

We have estimated the preservation of proteins in the burned bones and the possibility of their taxonomic diagnosis with anti-collagen antibodies. Taxonomic diagnosis of burned bones could be helpful for archaeologists in the reconstruction of technological behaviour. Results of our laboratory experiments show that a fraction of antigenic sites is preserved after one hour at temperatures below 400 °C, but is completely destroyed above this temperature although some protein traces are preserved (Figure 5).

Antigenic structures are also detected in extracts from bones burned at 550°C during a short period (< 3 min.). These limitations allow the use of antibodies for burned bones originating from the periphery of the fire place where the temperature is generally < 400°C or for the bone fragments burned during brief time intervals (e.g., cooking).

During fire experimentation, decrease of temperature was rapid for hearths with combustible bones, and some of the last bones added were partially unburned allowing for antigenic proprieties to be obtained as in unburned bone.

4. CONCLUSIONS

The best taxonomic specificity is within the family level which could be useful in some taxonomical diagnoses. The individual variability of the host animal's immunological reaction influences the taxonomic specificity of the reached antibodies. Several independent immunisation procedures are thus needed in order to obtain an *intra*-family taxonomic level.

Fossil collagen molecules generally show a decrease in their antigenic capacities when compared to their recent homologous ones. In our opinion, at least two factors are responsible to account for this loss of antigenicity. The first factor seems to be linked to the coating capacity of the fossil antigen on the polyvinyl plate (experimental artefact). In direct solid phase tests, fossil collagen molecules are therefore poorly recognised. This observation could be explained by the smaller size of such peptides which are not coated on the plate as well as the larger recent collagen peptides. On the other hand, these collagen molecules in liquid phase of a competition test inhibit the fixation of antibodies on the homologous recent collagen coated in solid phase. Direct and competition tests are thus needed to define the real antigenic properties of fossil collagen. The second factor leading to the decrease in antigenic potential corresponds to the disappearance of some antigenic sites which can not be reduced by increasing the amount of fossil antigen. The preferential disappearance of the specific terminal N and C

peptides could explain this observation and is in agreement with the theoretical model of collagen degradation (COLLINS *et al.*, 1995).

Antibodies can be raised against fossil proteins but this approach is restricted by two factors. Firstly, immunisations necessitate more antigens thus the destruction of a larger fossil sample. Secondly, immunisation against fossil proteins increases the possibility of non-specific antibodies obtained *via* taphonomic antigenic sites.

5. BIBLIOGRAPHY

ASCENZI A., BRUNORI M., CITRO G. and ZITO R., 1985,

Immunological detection of hemoglobin in bones of ancient Roman times and of Iron and Eneolithic ages. *Proceedings of the National Academy of Sciences. USA*, **82**: 7170-7172.

CANDELA P.B., 1936,

Blood-Group Reactions in Ancient Human Skeletons. *American Journal of Physical Anthropology*, **XXI**: 429-432.

CATTANEO C., GELSTHORPE K., PHILLIPS P. and SOKOL R.J., 1992,

Detection of Blood Proteins in Ancient Human Bone using ELISA: Survival of IgG and Albumin. *International Journal of Osteoarchaeology*, **2**: 103-107.

CATTANEO C., GELSTHORPE K., PHILLIPS P., WALDRON T., BOOTH J.R. and SOKOL R.J., 1995,

Diagnosis of multiple Myeloma in a medieval Bone. *International Journal of Osteoarchaeology*. **4**: 1-2

COLLINS M.J., RILEY M.S., CHILD A. and TURNER-WALKER G., 1995,

A Basic Mathematical Simulation of the Chemical Degradation of Ancient Collagen. *Journal of Archaeological Science*, **22**: 175-183.

GOODMAN M., 1962,

Evolution of the immunologic species specificity of human serum proteins. *Human Biology*, **34**: 104-150.

KOOYMAN B., NEWMAN M.E. and CERI H., 1992,

Verifying the Reliability of Blood Residue Analysis on Archaeological Tools. *Journal of archaeological Science*. **19**: 165-169

LOWENSTEIN J.M., 1980,

Species-species Proteins in Fossils, *Naturwissenschaften*. **67**: 343-346

LOWENSTEIN J.M., 1981,

Immunological reactions from fossil material. *Philosophical Transaction of the Royal Society London* B, **292**: 143-149.

SEMAL P., 1996,

Etude des propriétés antigéniques des collagènes osseux récents et fossiles. Thèse de doctorat, Université Libre de Bruxelles, 155 pp.

SEMAL P. and ORBAN R., 1995,

Collagen Extraction from Recent and Fossil Bones: Quantitative and Qualitative Aspects. *Journal of Archeological Science*, **22**: 463-467.

SMITH P.R. and WILSON M.T., 1990,

Detection of Haemoglobin in Human Skeletal Remains by ELISA. *Journal of Archaeological Science*, **17**: 255-268.

SHOSHANI J., LOWENSTEIN J.M., WALZ D.A. and GOODMAN M., 1985,

Proboscidean origins of mastodon and wooly mammoth demonstrated immunologicaly. *Palaeobiology*, **11**: 429-437.

ULRICH H.M.W., PERIZONIUS W.R.K., SPOOR C.F., SANDBERG P. and VERMEER C., 1987,

Extraction of osteocalcin from fossils bones and teeth. *Biochemical and Biophysical Research Communications*, **149**: 712-719.

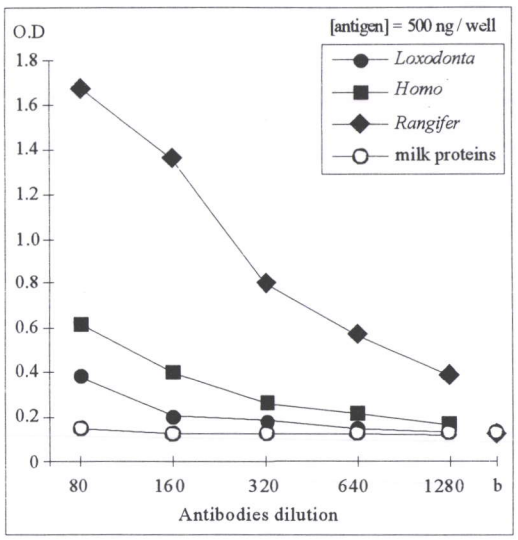

Figure 1 :
Fixation of hen's anti-reindeer antler collagen antibodies (dilutions 80 to 1280 X) on 500 ng of elephant collagen (*Loxodonta*), human (*Homo*) and reindeer (*Rangifer*) collagen or on milk proteins. O.D. = optical density at 495 nm. b = antibodies fixation on milk proteins (1%) used in order to block the free sites of the polyvinyl plate.

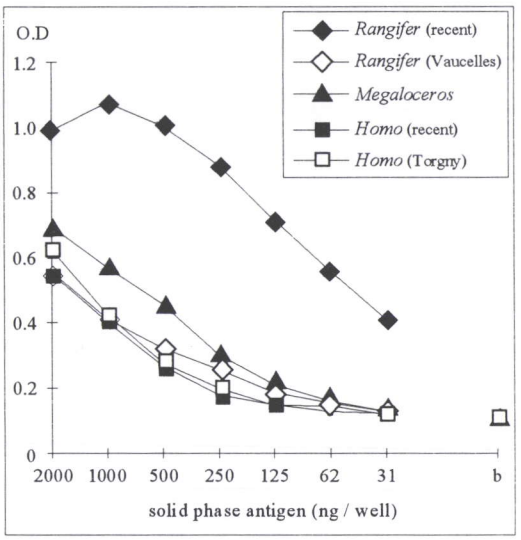

Figure 2 :
Direct fixation of hen's anti-reindeer antler collagen antibodies (diluted 160 X). Collagen in solid phase: recent reindeer, Upper Palaeolithic reindeer (Vaucelles), *Megaloceros*, recent and medieval (Torgny) human. O.D. = Optical density at 495 nm. b = antibodies fixation on milk proteins (1%) used in order to block the free sites of the polyvinyl plate.

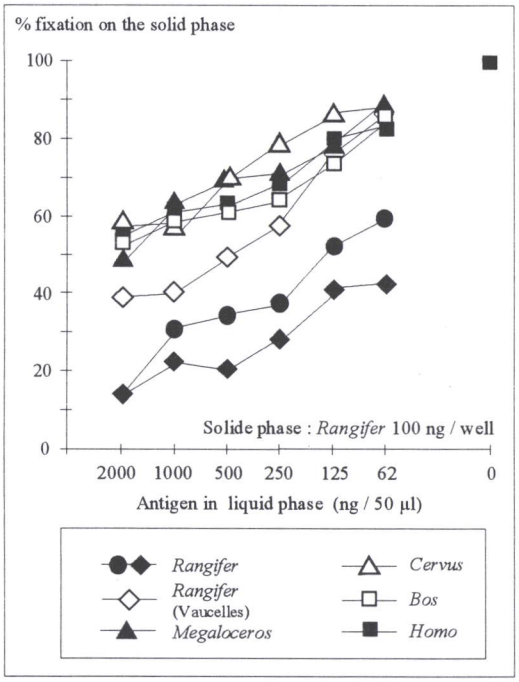

Figure 3 :
Competition test with hen's anti-reindeer antler collagen antibodies (diluted 120 X). Solid phase : recent reindeer antler collagen (100 ng / well). Collagens in liquid phase : recent reindeer (*Rangifer* 1 et 2), fossil reindeer (*Rangifer*, Vaucelles), fossil *Megaloceros*, recent red deer (*Cervus*), recent cow (*Bos*) and recent human (*Homo*). 0 = antibodies fixation without liquid phase

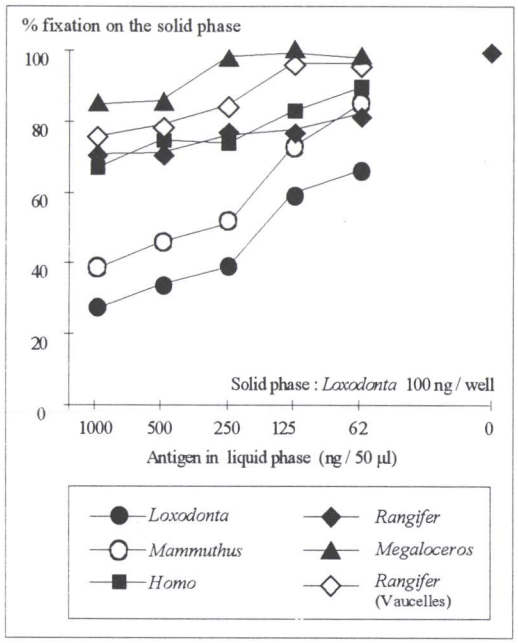

Figure 4 :
Competition test with hen's anti-mammoth collagen antibodies (diluted 160 X). Solid phase: recent elephant collagen (500 ng / well). Collagen in liquid phases : recent elephant (*Loxodonta*), fossil mammoth (*Mammuthus*), recent human (*Homo*), recent reindeer (*Ranfgifer*), fossil *Megaloceros* and fossil reindeer (*Rangifer* Vaucelles). 0 = antibodies fixation without antigens liquid phase.

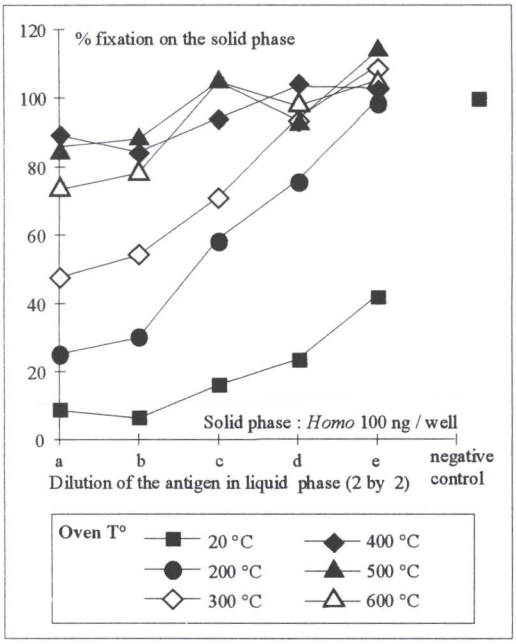

Figure 5 :
Competition test with rabbit anti-human collagen antibodies (diluted 100 X). Solid phase: recent human collagen (100 ng / well). Collagen in liquid phases : human collagen extracted from burned bones (20°C to 600 °C). Negative control = fixation of the antibodies on the solid phase (recent human collagen) without collagen in liquid phase.