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Preliminary report on a new composite material made of calcium phosphate, elastin peptides and collagens

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With a view to elaborating a bioactive bone substitute, the association of an artificial extracellular matrix, basically constituted of elastin-solubilized peptides (ESP) and type I + III collagens, with different types of calcium phosphates, was investigated. This paper describes the selective adsorption of ESP on some calcium phosphate samples, and the further association of the adsorbed peptides with type I + III collagens. A preliminary study of the material cytotoxicity was carried out, investigating the behaviour of human osteoblast cells in contact with the yielded composite material.

1. Introduction

Calcium phosphates, especially hydroxyapatite (HAP), have a composition and a structure very close to those of vertebrate bones and teeth [1]. In fact, they are often used as bone substitutes. Their biocompatibility is generally excellent and they may have a certain degree of bioactivity, as they appear to allow osteogenesis to occur [2]. However, these qualities, in particular their osteo-inductive capacity, can be further improved by associating a protein matrix to the mineral structure, and some recent investigations refer to the elaboration and bioactivity testing of such composite materials [3, 4].

Recently, a new reaction between elastin peptides and type I + III collagens has been described in our laboratory, yielding a crude connective matrix [5]. This substance has been markedly improved by adding connective or adhesive proteins and proteoglycans to yield artificial extracellular matrices: with heparan sulphate proteoglycan, it resembles more closely the natural subendothelial extracellular matrix [6, 7].

In other respects, one of the well-known properties of HAP is selectively to adsorb several proteins that are more- or less-tightly retained on the mineral network (adsorption chromatography). The objectives of the present investigations were to attempt to associate the new connective matrix to some calcium phosphate samples. This paper reports the ability of ESP to be tightly bound to several calcium phosphates where the product retains the ESP properties to react with type I + III collagens, leading to a composite material with organic and inorganic moieties. This composite material exhibited the ability to permit osteoblast activity in culture and, thus, might constitute a new approach to bone-substitute elaboration.

2. Materials and methods

2.1. Calcium phosphate samples

The experiments were carried out with different types of calcium phosphates prepared by mixing two solutions, one containing calcium and the other containing phosphate. The solutions were made up from stock solutions of calcium nitrate or chloride and of sodium or ammonium phosphate. The pH was brought to the desired value by the addition of either ammonia or sodium hydroxide. The differences in the conditions of synthesis imply differences in composition, structure and surface properties, as seen from Table I.

Samples T_0, T_1, T_2 and T_3 elaborated by Rey [1] correspond, respectively, to a β-tricalcium phosphate, a stoichiometric HAP, a non-stoichiometric HAP and finally an octacalcium phosphate of apatitic structure. The other samples elaborated by Lopez are all non-stoichiometric HAP, prepared at 37 °C according to the experimental conditions described above. The only difference between the preparations was the initial concentration of ammonia.

The microscopic aspect of the HAP-B_2 sample was observed by scanning electron microscopy (SEM) as shown in Fig. 1.

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TABLE I Characterization of calcium phosphate samples

Sample	X-ray diffraction crystallinity	Infrared spectroscopy, groups	Chemical analysis	Specific surface area ($m^2 g^{-1}$)
T_0	+ + +		Ca/p[a] = 1.5	4.4
T_1	+ + +	OH^-	Ca/p = 1.67	2.5
T_2	+	OH^-, CO_3^{2-}, HPO_4^{2-}	Ca/p = 1.51 CO_3^{2-} = 4.6%	7
T_3	+	OH^-, HPO_4^{2-}	Ca/p = 1.33	
B_1	+ +	OH^-, CO_3^{2-}, HPO_4^{2-}	Ca/p = 1.51 % CO_3^{2-}	93
B_2	+ +	OH^-, HPO_4^{2-}	Ca/p < 1.50	55
M_1	+ +	OH^-, $Ca(OH)_2$	Ca/p > 1.67	62
M_2	+ +	OH^-, $Ca(OH)_2$	Ca/p > 1.67	47

[a]p, phosphate.

2.2. Elastin-solubilized peptides

ESP were obtained by alkaline hydrolysis of insoluble elastin (Sigma) prepared according to a modification of the Jacob–Hornebeck method [8]. Briefly, 1 g elastin was suspended in 50 ml *t*-butanol (Merck) with gentle stirring; 50 ml 1 M KOH aqeous solution was added and the stirring continued until complete dissolution of elastin (48 h at 25 °C); 50 ml water was added and the clear solution was neutralized with acetic acid. The resultant solution was exhaustively dialysed against tap water overnight, then against three changes of doubly distilled water. Elastin peptides were then lyophilized, Rdt 80–90%. In use, ESP were dissolved in phosphate-buffered saline (PBS), pH 7.4. Whenever ^{125}I-labelled elastin was used, the solubilized peptides obviously conserved the labelling.

2.3. Type I + III collagens (at various ratios: 30–50% type III)

These were prepared by Institut Merieux (Marcy l'Etoile, France) from human placenta or by Bioetica (Lyon, France) from young bovine skin, using the classic pepsic digestion process followed by saline precipitation.

2.4. Buffers

PBS: 1 mM phosphate, 150 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, at pH 7.4 or 6.5.

Tris: 0.1 M tris(hydroxymethyl)aminomethane, 1 mM $MgCl_2$, at pH 8.5.

2.5. Fixation of ESP on calcium phosphates

Either ESP or type I + III collagens could be adsorbed on the calcium phosphates. For several reasons, mainly the protein conformation, less-elaborated in elastin peptides than in collagens, the association of ESP with the inorganic network was first investigated; then it was verified whether the reaction with collagens was possible. The association of ESP with calcium phosphate samples is usually effected by incubating 20 mg calcium phosphate in 1 ml buffered solution of ESP (10–20 mg ml^{-1}; PBS, pH 7.4). The mixture was then incubated at 37 °C, with gentle stirring, for 1 h. The reaction was stopped by centrifugation (5000 r.p.m. for 10 min) and finally the pellets were washed twice with 1 ml PBS, pH 7.4 at 37 °C with stirring, for 15 min each time. In order to study this association, ^{125}I-labelled ESP was used; the amount of peptides retained on calcium phosphate was expressed as a percentage of the radioactivity.

Figure 1 Microscopic feature and size of calcium phosphate B_2 particles.

2.6. Association of type I + III collagens with HAP–ESP

The HAP–ESP material was prepared as described above. The washed pellets were then resuspended in 1.2 ml PBS, pH 7.4 and 0.8 ml type I + III collagen solution (10 mg/ml in H_2O). After a short but effective

homogenization, the mixture was incubated at 37 °C until coagulation of the system occurred.

2.7. Scanning electron microscopy

The material was completely dehydrated and a thin layer of gold–palladium was deposited on the samples, which were examined in an SEM (Hitachi, model S-2500) operated at 20 kV. Photomicrographs were taken at magnifications ranging from ×80 to ×30 000.

2.8. Materials for *in vitro* biocompatibility tests

Iscove's modified Dulbecco's medium (IMDM) was obtained from Gibco; foetal calf serum (FCS), PBS and trypsin from Institut Jacques Boy. 3-(4,5-dimethiazol-2-yl)-2,5-diphenyl tetratolium bromide (MTT) powder, dimethyl siloxane (DMSO) and the kit for the histochemical demonstration of alkaline phosphatase (85L-2) were purchased from Sigma, and Neutral red from RAL-Kuhlmann. Culture flasks (25 cm^2) were from Corning; four- and 96-well plates (15.5 and 6.4 mm-diameter wells, respectively) and Lab-Tek chamber slides were from Nunc.

2.9. Alveolar bone osteoblast cultures

For the *in vitro* biocompatibility tests, alveolar bone osteoblasts, in the third passage, were used. These differentiated cells were obtained by the explant method as described in [9], using tissue samples from a healthy 18-year-old volunteer undergoing endodontic surgery.

Briefly, 10 explants (about 1 mm^3), carefully washed in order to remove as much marrow as possible, were placed on the bottom of 25 cm^2 culture flasks and left to attach for 3 h at room temperature before the addition of 5 ml culture medium [IMDM supplemented with 10% (v/v) FCS]. No antibiotic was used. Incubation was performed at 37 °C. After 8 days the medium was changed for the first time, and thereafter the cultures were refed every 7 days. Cell outgrowths from the explants were observed 3–4 weeks later. When confluency was reached, subcultures were realized by detachment of the cell layer with trypsin [0.2% (w/v) in PBS, Ca^{2+}- and Mg^{2+}-free].

2.10. Extraction liquid preparation

The extraction liquid of the material was prepared according to the S-90-701 AFNOR standard [10]. The extraction was carried out with the culture medium IMDM + 10% FCS, with a surface rate of 5 $cm^2 ml^{-1}$, under sterile conditions and soft contraints (37 °C, 120 h, without stirring).

An "extraction blank", i.e. the growth medium subjected to the same conditions but without the material, was prepared simultaneously.

2.11. Neutral red test

The osteoblasts were seeded into the 6.4 mm-diameter wells (10^4 cells $well^{-1}$) in IMDM + 10% FCS, and then incubated (37 °C, humidified atmosphere containing 5% CO_2) until confluency was reached. Four concentrations of the extraction liquid and of the extraction blank, prepared in IMDM + 10% FCS 100, 50, 10 and 1%, were distributed at 200 µl $well^{-1}$, eight wells per concentration. The plates were then incubated for 72 h (37 °C, 5% CO_2). After washing the cells with PBS, 200 µl medium containing 50 µg ml^{-1} Neutral red were added to the wells. The Neutral red stain was fixed by electrostatic binding on the anionic sites of the lysosome matrix. An alteration in this membrane, due to a cytotoxic effect of the extraction liquid, decreases the stain fixation. The coloration needed a 3 h incubation at 37 °C. After this time the medium was eliminated and substituted first by a 10% formaldehyde solution for 15 min and secondly by a 10% acetic acid solution for 30 min. After homogenization of the coloration the plates were read by an automatic analyser (λ = 540 nm). The results were finally expressed as percentage mortality.

2.12. MTT test

Another test previously described by Mosmann *et al.* [11] was also carried out for the evaluation of the extraction liquid cytotoxicity. MTT, is a water-soluble tetrazolium salt that is metabolized in the mitochondria by the succinate-deshydrogenase of living cells to form blue formazan crystals.

The first steps were equivalent to those of the Neutral red test. Once the cells had been incubated for 72 h in the different concentrations of the extraction liquid and blank, they were carefully washed to eliminate the culture medium completely. Then, 125 ml MTT solution (1 mg/ml in Hanks' buffer containing 1 g ml^{-1} glucose) were distributed. The plates were incubated for 3 h (37 °C and 5% CO_2). During this time the formazan crystals were formed, only in living cells. They were solubilized by the addition of a DMSO solution at 100 µl $well^{-1}$. After a quick homogenization the plates were read by an automatic analyser (λ = 540 nm). The final results were also expressed as percentage mortality.

2.13. Histochemical visualization of alkaline phosphatase activity

Osteoblasts were seeded in Lab-Tek wells (10^4 cells $well^{-1}$). The alkaline phosphatase activity was revealed histochemically by the azo-dye method of Ackerman [12] using the Sigma diagnostic kit (85L-2). Cells were fixed for 30 s at room temperature in 60% citrate-buffered acetone. The substrate was naphthol AS-MX phosphate. As a result of phosphatase activity, naphthol AS-MX was liberated and immediately coupled with a diazonium salt (Fast Blue RR salt), forming an insoluble blue pigment at sites of alkaline phosphatase activity. One hundred consecutive cells were observed: rates from 0 to 4^+ were evaluated according to Kaplow [13] on the basis of the quantity and staining intensity of precipitated dye within the cytoplasm of the cells.

2.14. Elaboration of capsules for cell culture support

The composite material was elaborated as described above, and rapidly distributed at 400 μl well^{-1} (15 mm diameter). The plates were incubated at 37 °C for 1 h, then left to desiccate slightly for 24 h before their sterilization by 25 kG gamma-ray treatment.

2.15. Osteoblasts biointegration study: SEM observations

The osteoblasts were seeded (15×10^3 cells well^{-1}, 15 mm diameter wells) in both capsule-containing wells and in control wells. Twenty-four hours after seeding the supernatants were screened for cells (Malassez cell) and the seeded capsules and control wells were prepared for SEM observation: first, the cells were fixed in glutaraldehyde–cacodylate buffer, then washed in 0.15 M cacodylate solution, completely dried at room temperature, and finally a thin layer of gold–palladium was deposited on the samples.

3. Results

3.1. Adsorption of ESP on calcium phosphate samples

Different types of calcium phosphates, previously washed and equilibrated in PBS, pH 7.4, were used. Their different reactivities as regards the ^{125}I-ESP adsorption has been the criterion for the choice of two calcium phosphate samples with which the biochemical study of the reaction was carried out. The retained radioactivity of the crude and washed pellets was measured for all samples. As shown in Table II, sample B_2 retained the highest level of radioactivity, that is the amount of elastin solubilized peptides, followed by sample B_1. Both were HAP prepared by the same method, at 37 °C, but at different initial pH (7.9 and 11.3, respectively).

3.2. Biochemical studies of the ESP adsorption

3.2.1. pH

HAP is commonly used as a support for adsorption chromatography, because of its capacity to adsorb several proteins selectively. As this binding depends strongly on the pH, we performed the ^{125}I-ESP adsorption on samples B_1 and B_2 with buffers at different pH: PBS 6.5, PBS 7.4 and Tris–HCl 8.6. For both samples the optimal pH for the reaction was 7.4, as illustrated in Fig. 2.

TABLE II Percentages of retained radioactivity in pellets

Sample	Crude pellets	1st washing	2nd washing
T_0^a	9.5	4.6	3.5
T_1^a	7.1	3.9	3.3
T_2^a	26.1	21.4	20.8
T_3^a	24.9	20.3	19.6
B_1^b	34.1	28.5	25.3
B_2^c	37.7	32.1	32.8
M_1^c	29.9	25.7	23.0
M_2^a	25.8	22.6	20.1

[a] One result, [b] mean of three results, and [c] mean of two results.

3.2.2. Kinetics

The study of ^{125}I-ESP adsorption on calcium phosphate samples as a function of time allows the determination of the maximal percentage of retained radioactivity and the time needed to reach the plateau. The radioactivity retained by the pellets, unwashed and washed, obtained at different times of the kinetic study, was read. In Fig. 3 the results show that not only that sample B_2 had a higher adsorption capacity, confirming the later results, but also that it adsorbed the ^{125}I-ESP more rapidly. The plateau was reached after 1 h incubation, with a percentage of retained radioactivity of about 25–30%.

3.2.3. Stoichiometry

With a view to elaborating samples of composite material (which have to be homogeneous and dense)

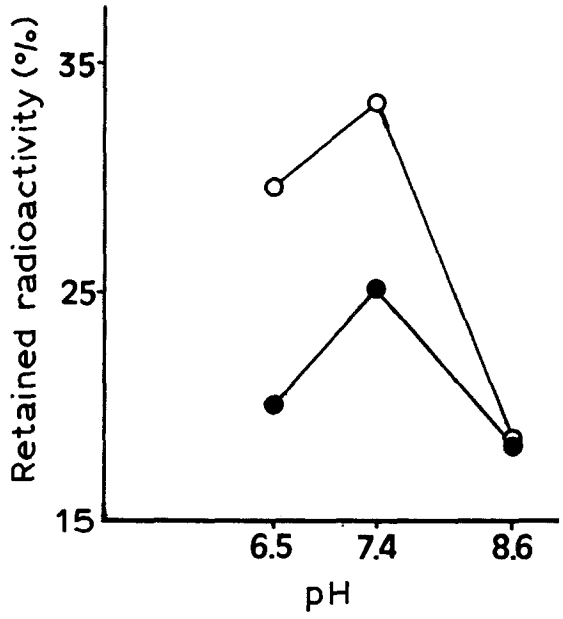

Figure 2 ESP adsorption versus pH: (●) B_1 and (○) B_2.

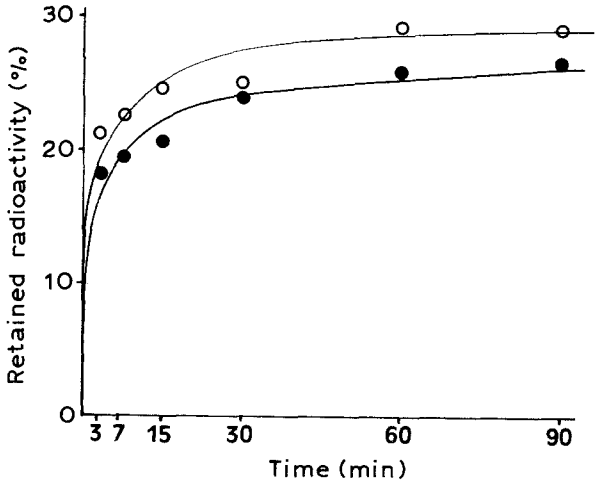

Figure 3 Kinetics of ESP adsorption: (●) B_1 and (○) B_2.

TABLE III Stoichiometric study: variation in HAP amount

	HAP (mg)					
	5	10	20	40	50	60
^{125}I-ESP, 20 mg ml^{-1} (ml)	0.5	0.5	0.5	0.5	0.5	0.5
PBS, pH 7.4 (ml)	0.5	0.5	0.5	0.5	0.5	0.5

TABLE IV Stoichiometric study: variation of ^{125}I-ESP concentration

	HAP (mg)						
	20	20	20	20	20	20	20
^{125}I-ESP (ml)	0.05	0.1	0.2	0.4	0.6	0.8	1
(mg)	1	2	4	8	12	16	20
PBS, pH 7.4 (ml)	0.95	0.9	0.8	0.6	0.4	0.2	

the optimal relative ratio of the reactants must be determined. Therefore, the reaction of a given concentration of ^{125}I-ESP on several amounts of HAP, then the reaction of various concentrations of ^{125}I-ESP on 20 mg HAP, were carried out, as shown in Tables III and IV.

According to the thermodynamic and kinetic conditions used, the perfect relationship between both situations should be noted (Fig. 4): whenever we had a ^{125}I-ESP solution of 10 mg ml^{-1} reacting with 20 mg HAP, the amount of peptides retained was approximately the same (2.6–2.7 mg). This result emphasized the fact that only some peptides are retained on HAP. This was corroborated by saturating a given amount of HAP with repeated concentrations of ESP: for 20 mg HAP, five times 1 ml ^{125}I-ESP (10 mg ml^{-1}) were necessary to obtain total saturation, i.e. about 0.3 mg peptides mg^{-1} HAP (Fig. 5).

3.3. Association of type I + III collagens with HAP–ESP

The HAP–ESP material was prepared as described above. After 4 min incubation the suspension was coagulated; the pellet was desiccated for SEM. As illustrated in Fig. 6, SEM visualization of the composite material showed the heterogeneity of the surface; but each grain was closely covered with the organic fibrillar matrix, not only on the surface but all around the particles. The reaction between HAP–ESP and collagens is a little delayed, inducing a light decantation which produces as a consequence a thicker layer of collagens on the top surface.

3.4. Elaboration of capsules for cell culture support

The capsular material was elaborated, as described above, and left to desiccate slowly at room temperature for about 3 days. Unfortunately, the yielded capsules were shrunk and thus cracked even if they adhered quite well to the bottom and walls of the well (Fig. 7a).

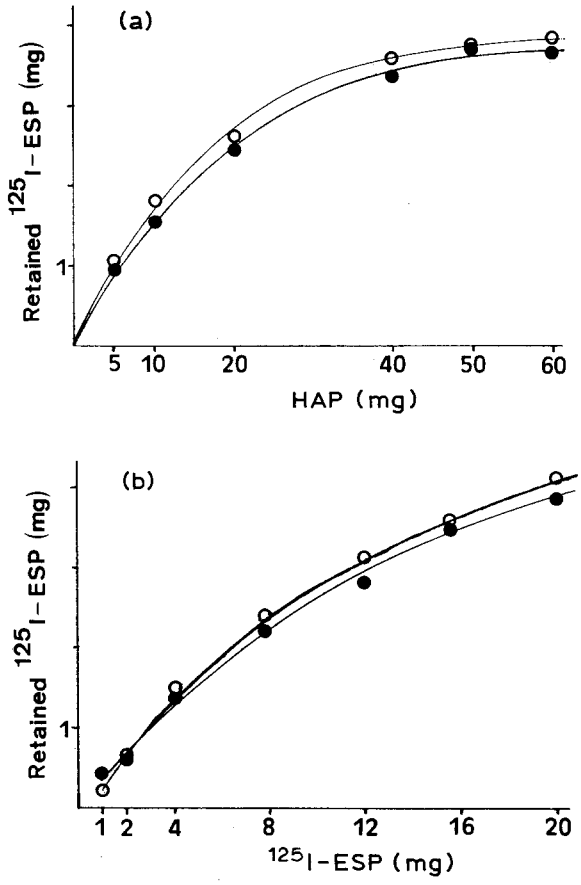

Figure 4 Stoichiometric studies: (a) variation in retained ^{125}I-ESP versus HAP amount and (b) variation in retained ^{125}I-ESP versus ESP concentration. (●) B_1 and (○) B_2.

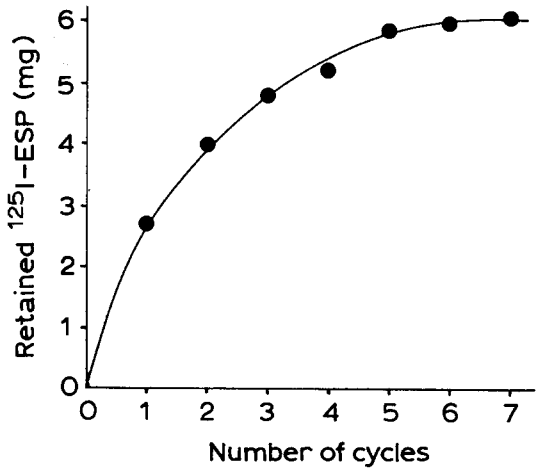

Figure 5 Saturation study: determination of total amount of ESP that can be adsorbed on HAP (sample B_1, see description of the cycle in text).

Neither the increased amount of either ESP or collagens, nor the increased amount of HAP greatly improved the structural quality of the material, although in the latter case there was a slight improvement (Fig. 7b). Another way to improve the capsule quality was to add an aliquot of elastin powder in the material during its elaboration. However, this required too great a quantity of elastin for a not too good result (Fig. 7c). In order to prepare homogeneous and dense capsules, suitable to promote cell cultures, the best temporary solution was not to

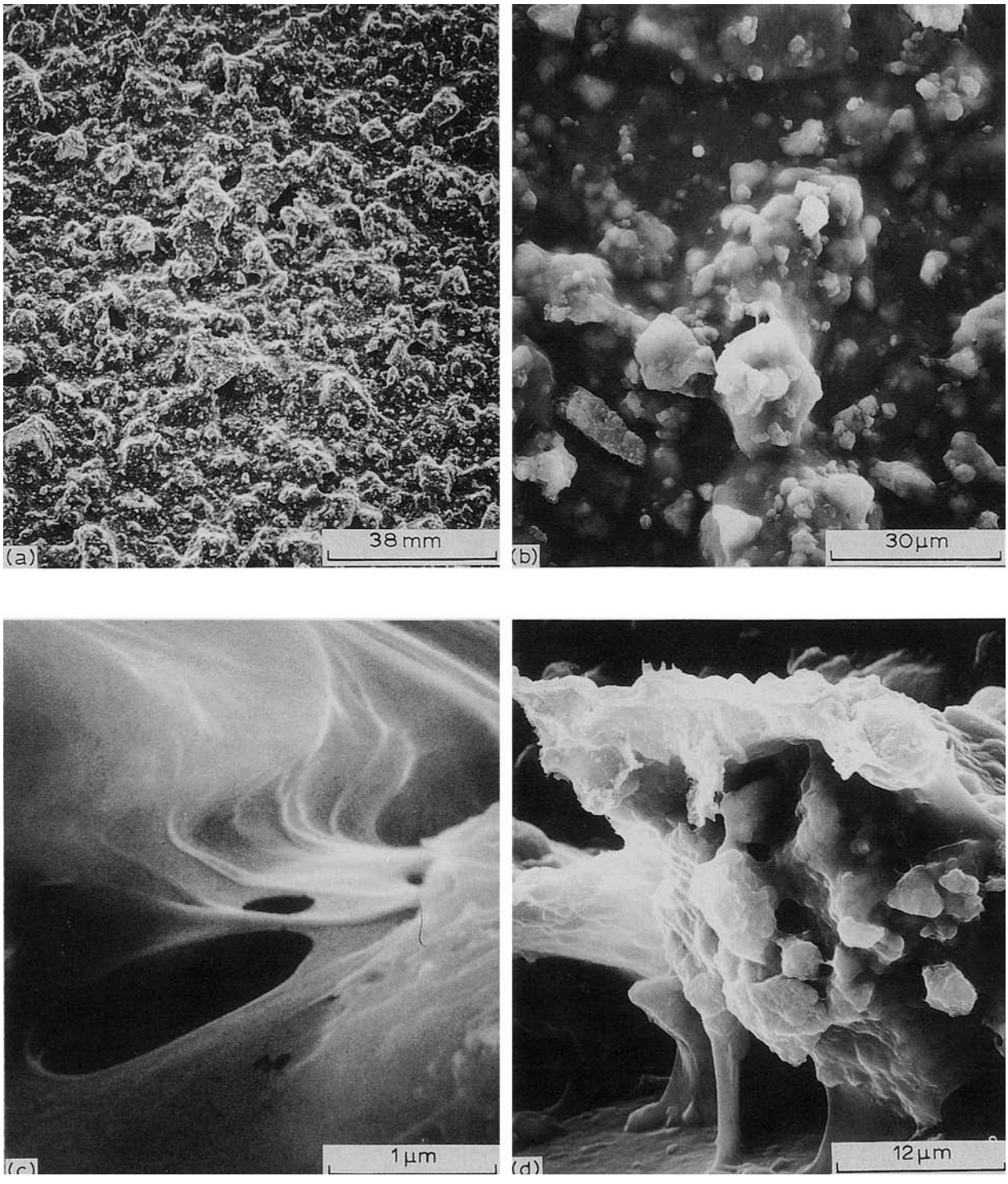

Figure 6 SEM visualization of the composite material HAP–ESP collagens: (a) surface of the material, (b) surface of the material, (c) surface of the material and (d) transverse section of the material.

desiccate the material completely, leaving it slightly wet (Fig. 7d); this led us to check, by SEM examination, that γ-ray treatment (25 kGy) did not damage the material. No disorder was apparent on the structure.

3.5. Preliminary study of *in vitro* biocompatibility

3.5.1. Cell viability: Neutral red and MTT tests

The cytotoxicity of the material extraction liquid was determined by the incorporation of a vital stain, Neutral red or MTT. Certainly, the spectrophotometric evaluation of the coloration, either produced by the retention of Neutral red stain or by the formation of Formazan crystals (Fig. 8), is a good indication of the number and the metabolic activity of living cells.

It is normally accepted that a mortality of $> 20\%$ indicates that the tested material is cytotoxic. For both tests the results showed a slight toxic effect of the undiluted extraction liquid (100%) in comparison with the undiluted extraction blank as seen from Table V. Conversely, the three tested extraction liquid dilutions (50, 10 and 1%) were not cytotoxic for the osteoblasts in culture.

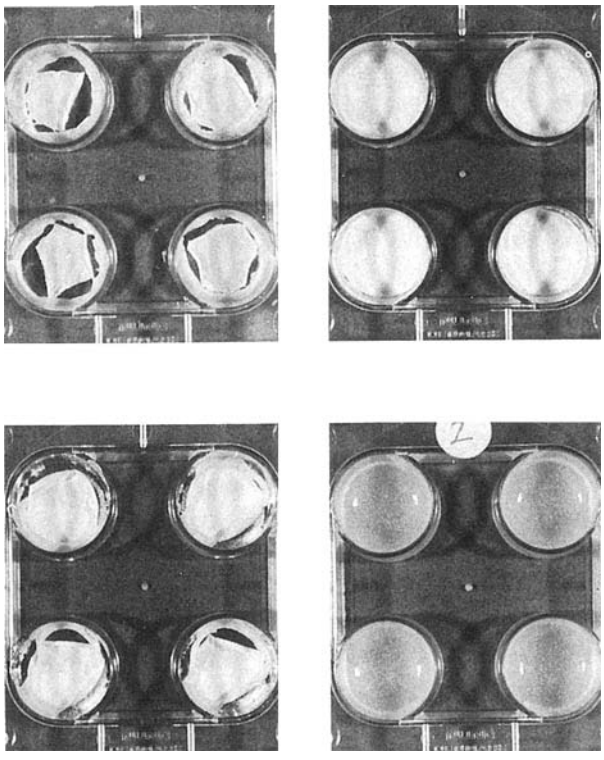

Figure 7 Elaboration of composite material capsules (see text).

TABLE V Cell mortality percentages

	Extraction liquid concentration (%)			
	1	10	50	100
Mortality, Neutral red test	1.3	5.4	6.3	27.7
Mortality, MTT test	10.1	15.3	− 4.5	− 25.5

3.5.2. *Alkaline phosphatase intracellular activity*

The cytotoxic effect of a material may not modify only cell viability, but also cell differentiation. One specific characteristic of osteoblasts is their high alkaline phosphatase intracellular activity. Therefore, the evaluation of this activity by a histochemical method will also be an indication of the potential cytotoxicity of the material tested. Here again, the slight cytotoxic effect of the undiluted extraction liquid (100%), as seen above for the cell viability test, is observed: 70% of osteoblasts cultivated in 100% extraction liquid were negative or very slightly positive cells, but 70% of osteoblasts cultivated in 100% extraction blank were well-defined to strongly positive cells.

3.5.3. *Osteoblast culture on the composite material: SEM observations*

A preliminary study of osteoblast behaviour on the material was carried out by SEM observations of cell attachment and spreading on the material surface. Twenty-four hours after seeding on the material capsules and on control wells, the supernatants were screened for cells in a Malassez cell; no cells were

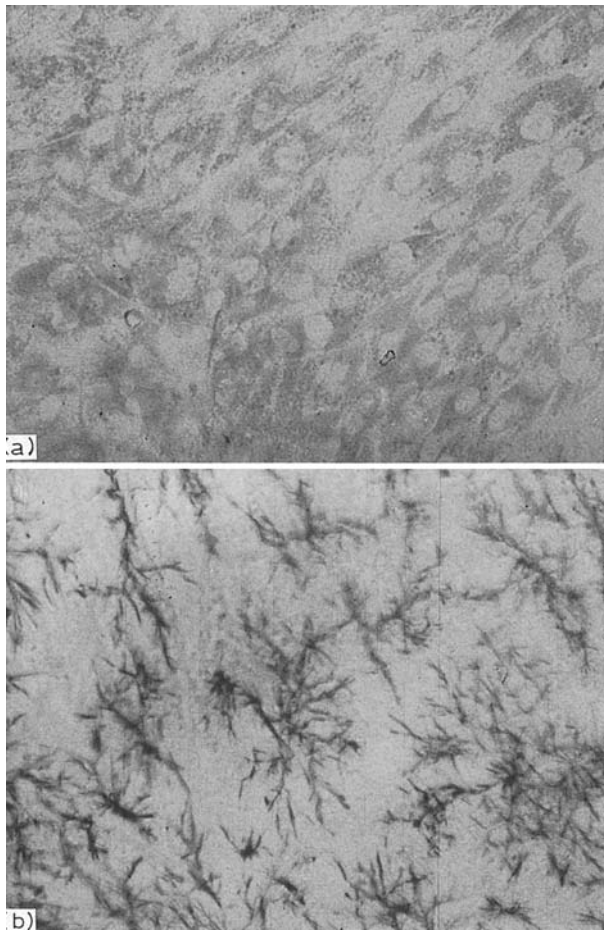

Figure 8 Test of cytotoxicity: (a) Neutral red stained osteoblasts and (b) formazan crystals formed by living osteoblasts (MTT test).

observed. The seeded capsules and control wells were then prepared for SEM observation; the osteoblasts were well attached and spread on the material surface, and their morphology was multipolar and rich in cytoplasmic extensions (Fig. 9).

4. Discussion

The use of biomaterials in surgery increasingly requires higher material quality, especially with regard to their biocompatibility, and the search for bioactive materials is now widely under way. The objectives of this work were the conception, the preliminary assays of elaboration and the characterization of a potential biomaterial which could possibly be used in surgery as a bone substitute.

The ideal material must be elaborated in such a way that it induces completely normal reactions from the living tissues with which it is in contact. As a consequence, it must be not only biocompatible but also bioactive. Therefore, the elaboration of a material with a structure and composition similar to those of bone, i.e. a material resulting from a tight association of an organic component with a mineral, would be of great interest.

The collaboration between laboratories specializing in calcium phosphate studies or competent in the field of artificial connective matrices has allowed us to elaborate an original hard connective matrix. This

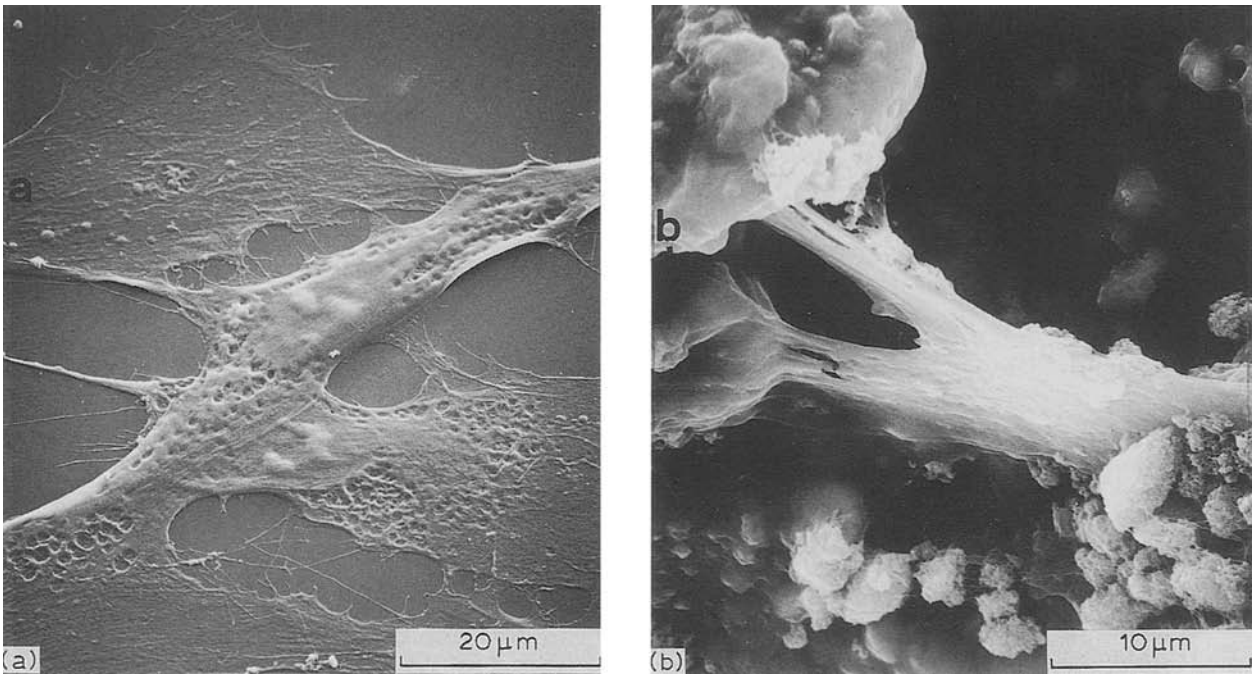

Figure 9 SEM visualization of osteoblasts: (a) on polystyrene, control culture and (b) on the composite material.

new composite material results from the irreversible binding of proteins to calcium phosphates, in particular to HAP.

Several calcium phosphate samples were assessed. Their degree of performance in relation with the ESP adsorption has been the criterion that determined the choice of sample B_2, a non-stoichiometric HAP, containing hydrogenophosphate ions, and synthesized in physiological conditions. The HAP capacity of adsorbing molecules is a well-known phenomenon used, for example, in adsorption chromatography. However, the nature of this adsorption is still the subject of many investigations. It is probably due to the attraction of opposite charges, where the HAP charged groups, especially calcium and orthophosphate ions, play an important role [14]. The specific surface values of the different calcium phosphate samples tested, and their content of charged groups, could be the characteristics that determine the capacity of ESP binding. Of course, the peptides characteristics, in particular their isoelectric pH, are also involved.

From the biochemical studies carried out, it is notable that the ESP adsorption depends on the type of calcium phosphate, is irreversible and is selective. The adsorbed ESP conserved their reactivity with the collagens; however, we note that this reaction is noticeably delayed when the ESP are adsorbed. Although the nature of the reaction between ESP and collagens is not yet determined, we can propose two hypotheses to explain this delay. On the one hand, the adsorbed peptides certainly undergo loss of charges, which modifies their molecular conformation and, therefore, causes a decrease in their reactivity (number of disponible reactive sites, etc.). On the other hand, the presence of HAP can also be the cause of this delay, since a recent ultraviolet spectroscopy study (unpublished work) shows the inhibition effect of calcium ions on the reaction between ESP and collagens.

The cell viability tests show that the 100% extraction liquid is cytotoxic. This decrease in cell viability is accompanied by an effect on the specific phenotype of osteoblasts. Nevertheless, when the extraction liquid is diluted in culture medium, the results of the three tests are good; they are under the normal accepted limits for toxicity. These tests seek to determine the possible cytotoxic effect of the material on the cells "in culture". Therefore, the importance of the cytotoxicity due to the 100% extraction liquid must not be overestimated: one of the main characteristics of these *in vitro* tests of a material's biocompatibility in comparison with the *in vivo* tests, is the higher sensitivity registered by the cells in culture [15]. The main interest of using cell cultures is the possibility to observe the behaviour of specific cell types in a well-controlled environment. Therefore, the action of a biomaterial on the cell types, specifics of the implantation site, must be studied [15, 16]. However, *in vivo* the cells never act as isolated. The tissular reaction to implanted biomaterials is a series of very complex responses that may take place over several months. Consequently, the conditions that can be reproduced in cultures are only a simplified part of what happens *in vivo* [15]. The particular reaction of the isolated cells (to the 100% extract) would probably be an example. The sensitivity of this method is surely an advantage, but it must be taken into account that the *in vitro* observed toxicity is certainly an overestimate of the *in vivo* toxicity, due to the particular conditions of the *in vitro* system. We must emphasize, for example, that the cells in culture are isolated from the excretion and detoxification ways.

The osteoblasts attachment and spreading on a particular support is an indispensable requisite for their proliferation and, therefore for the synthesis of the osteoid matrix and its mineralization. Therefore, our preliminary results are encouraging. Another ex-

perience, over a longer period, revealed that the material was disorganized and partially lysed on the 10th day. This degradation may be due to a degrading action of osteoblasts. Actually, Gregoire *et al.* [17] showed that osteoblasts have the capacity to phagocyte the calcium phosphate particles. Another possible explanation would be the action of the culture medium as an "extraction vehicle". Some complementary experiments are in progress.

Nevertheless, this study clearly demonstrates the ability of some HAP to be tightly associated with connective matrix. The yielded material reveals some preliminary interesting properties related to osteoblast culture, in spite of some problems due to an eventual toxic effect. However, several investigations are in progress in our laboratory: first, to discriminate the ideal HAP sample; secondly, to improve the quality of the associated matrices; and thirdly, to determine extensively biochemical and biological reactions induced by the material in systemic and localized conditions. Therefore, direct methods for testing basal and specific biocompatibility as well as bio-integration will be considered first *in vitro*, then in animals.

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References

1. C. REY, *Biomaterials* **11** (1991) 13.
2. G. DACULSI and N. PASSUTI, in "Bioceramics II", edited by H. Onishi and Hemcke (Ishigakuro, Tokyo, 1991) pp. 345–354.
3. A. SUGAYA, M. MINABE, T. TAMURA, T. HORI and Y. WATANAVE, *J. Periodont. Res.* **24** (1989) 284.
4. D. A. GLASS, J. T. MELLONIG and H. J. TOWLE, *ibid.* **60** (1989) 121
5. M. RABAUD, F. LEFEBVRE and D. DUCASSOU, *Biomaterials* **12** (1991) 313.
6. F. LEFEBVRE, S. GORECKI, R. BAREILLE, J. AMEDEE, L. BORDENAVE and M. RABAUD, *ibid.* **13** (1992) 28.
7. L. BORDENAVE, F. LEFEBVRE, R. BAREILLE, F. ROUAIS, C. BAQUEY and M. RABAUD, *ibid.* **13** (1992) 439.
8. M. P. JACOB and W. HORNEBECK, in "Frontiers of Matrix Biology", vol. 10, edited by L. Robert (Créteil, Karget, Basel, 1989) p. 92.
9. M. F. HARMAND, L. BORDENAVE, R. DUPHIL and D. DUCASSOU, *Calcif. Tissue Int.* **36** (1984) 29.
10. ASSOCIATION FRANÇAISE DE NORMALISATION (AFNOR), COMISSION "BIOCOMPATIBILITÉ DES IMPLANTS CHIRURGICAUX", "Matériel Médico-chirurgical. Biocompatibilité des Implants Chirurgicaux. Méthodes d'Extraction", S93F, Dec. 12, Pr. S. 90.701 (April 1977).
11. T. MOSMANN, *J. Immunol. Methods* **65** (1983) 55.
12. G. A. ACKERMAN, *Lab. Invest.* **11** (1962) 563.
13. L. S. KAPLOW, *Ann. NY Acad. Sci.* **155** (1968) 911.
14. G. BERNARDI and T. KAWASAKI, *Biochim. Biophys. Acta* **160** (1968) 301.
15. C. J. KIRKPATRICK and C. MITTERMAYER, *J. Mater. Sci. Mater. Med.* **1** (1990) 9.
16. T. RAE, in "Technics of Biocompatibility Testing", edited by D. F. Williams (CRC Press, Boca Raton, Florida, 1986) p. 81.
17. M. GREGOIRE, I. ORLY and J. MENANTEAU, *J. Biomed. Mater. Res.* **24** (1990) 165.

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