SPECIFIC LOCALIZATION AND IMAGING OF AMYLOID DEPOSITS IN VIVO USING ^{123}I-LABELED SERUM AMYLOID P COMPONENT

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Systemic amyloidosis is a serious clinical disorder for which there is no effective treatment (1). It is characterized by the widespread extracellular deposition of autologous protein in an insoluble fibrillar form (2, 3). The deposits relentlessly accumulate and persist in the tissues, usually resulting in organ dysfunction and death within 1–5 yr of diagnosis. The presence of amyloid can only be confirmed by histological examination of tissue obtained by biopsy or surgical resection. These procedures cannot provide information on the presence and distribution of amyloid in the body as a whole, and in consequence, little is known about the natural history of this condition.

A wide variety of precursor proteins can form amyloid fibrils, notably Ig light chains in amyloid of Ig light chain type, amyloid A protein (AA)[1] in reactive systemic amyloid, and prealbumin in familial amyloid polyneuropathy and senile systemic amyloid (1–3). In addition, a nonfibrillar glycoprotein, amyloid P component, is present as a minor constituent in all forms of amyloid deposit (4, 5), with the single exception of the intracortical plaques of Alzheimer's disease and senile dementia of Alzheimer type (6–8). We have previously demonstrated that amyloid P component is derived from the normal plasma protein, serum amyloid P component (SAP) (9). SAP, together with C-reactive protein (CRP), the classical acute-phase reactant, is a member of the pentraxin family of plasma proteins, so named because of their molecular appearance as pentameric discs in the electron microscope (10, 11). SAP undergoes calcium-dependent binding to amyloid fibrils in vitro (12) and presumably this is the mechanism by which plasma SAP that has left the circulation deposits with amyloid fibrils in vivo (9).

We now report the use of isolated purified SAP as a specific in vivo targeting vehicle for amyloid. These studies used radiolabeled protein and gamma camera imaging in mice with experimental amyloidosis, but they establish the important principle of the use of this approach which has implications for diagnosis and, perhaps, ultimately treatment of human amyloidosis.

This work was supported in part by Medical Research Council Programme Grant G979/51 to M. B. Pepys. P. N. Hawkins is a Medical Research Council Training Fellow.

[1] *Abbreviations used in this paper:* AA, amyloid A protein; AEF, amyloid-enhancing factor; CRP, C-reactive protein; SAP, serum amyloid P component.

Materials and Methods

Mice. Female CBA/Ca mice, 8–20 wk old, were obtained from the National Institute for Medical Research (London).

Induction of Amyloid. For the imaging experiments, amyloidotic mice were produced by daily subcutaneous injection of 0.5 ml vitamin-free casein (ICN Pharmaceuticals Inc., Cleveland, OH) at 10% wt/vol in 0.05 M $NaHCO_3$ (13) five times per week for 5 wk. Amyloid was induced in mice used for ^{125}I trace labeling experiments by intravenous injection of 0.5 ml amyloid-enhancing factor (AEF), followed immediately by subcutaneous injection of 0.5 ml 2% wt/vol $AgNO_3$ in distilled water. The animals were studied 4 wk afterwards. AEF was prepared by the method of Axelrad et al. (14) using the spleens of CBA mice in which amyloidosis had been induced by repeated casein injections.

Histology. Amyloid was identified by alkaline alcohol Congo red staining (15) of 6-μm frozen sections and the extent of deposition was estimated semiquantitatively by determination of the proportion of the area of the sections occupied by Congophilic green birefringent material.

RIA for Mouse AA in Tissue. AA was quantitated by RIA in standard homogenates of spleen and liver that had been treated with formic acid to solubilize amyloid fibrils and enhance their antigenic reactivity. The RIA (Hawkins, P. N., and M. B. Pepys, manuscript in preparation) involved absorption of a fixed dose of rabbit anti-AA antibodies (produced by immunization with acute-phase mouse HDL [16]) by the tissue AA. Residual anti-AA activity was then measured in a microtiter plate solid-phase system with degraded AA amyloid (17) coated on the wells and ^{125}I-labeled immunopurified goat anti-rabbit IgG antibody for detection of bound anti-AA. The assay was standardized using dilutions of a homogenate of a pool of very heavily amyloid-laden spleens and results are expressed as percent equivalence to this AA-rich standard per gram of whole organ.

Proteins. Mouse SAP (18), human SAP (19), human CRP (19), and *Limulus polyphemus* CRP (20) were isolated and purified as previously reported. All preparations were >99% pure by reduced SDS-PAGE (21) with heavily overloaded gels (50–100-μg samples) stained with Coomassie Blue.

Iodination of Proteins. Purified proteins were iodinated by the *N*-bromosuccinimide (British Drug Houses, Poole, England) method (22) and then separated from free iodide by gel filtration on Sephadex G25 (PD10 column; Pharmacia Ltd., Milton Keynes, UK) in PBS. For RIA of AA in tissues and trace labeling experiments with pentraxins, carrier-free $Na^{125}I$ (IMS 30; Amersham plc, Amersham, UK) was used. For imaging experiments, dry $Na^{123}I$ (Atomic Energy Research Establishment, Harwell, UK) was used. After gel filtration, >95% of the radioactivity was precipitable with 10% wt/vol TCA. Functional integrity of labeled pentraxins was confirmed in each case by demonstrating that they retained intact their specific ligand-binding activity for phosphoethanolamine immobilized on carboxyhexyl Sepharose (Pharmacia Ltd.) (23).

Gamma Camera Imaging and Kinetics of Localization of ^{123}I-SAP. Human SAP: groups of six mice, three with casein-induced amyloid and three controls maintained under otherwise identical conditions, were injected intravenously with 550 μCi ^{123}I-human SAP (sp act, 5.5 μCi/μg) at time 0 and were scanned at 0.5, 2, 4, 19, and 24 h thereafter with a Toshiba gamma camera and pinhole collimator, for periods of 1–4 min under ether anesthesia. The mice were then bled out and killed. The TCA-precipitable cpm/g of blood were determined and the spleens, livers, and kidneys were removed and counted in a gamma counter. The organs were then snap frozen for histological processing.

Mouse SAP: the same procedure was performed using 550 μCi ^{123}I-mouse SAP (sp act, 5.5 μCi/μg) except that the scans were performed at 0, 0.5, 3, 5, and 24 h. All counts were corrected for isotope decay.

Organ Localization of ^{125}I-Labeled Proteins. Four groups of 10 mice, five with AEF-induced amyloid and five controls, were injected intravenously with the following radiolabeled proteins respectively: ^{125}I-mouse SAP, ^{125}I-human SAP, ^{125}I-human CRP, and ^{125}I-*Limulus* CRP. Each mouse received 0.6 μCi of ^{125}I-protein at time 0. The specific activity of each protein was 0.4 μCi/μg. At 24 h the mice were bled out, and their spleens, livers, and kidneys were removed, weighed, and counted in a gamma counter. A portion of each

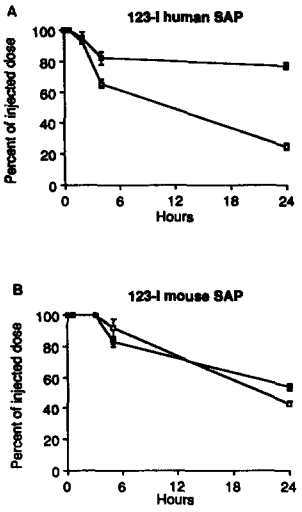

FIGURE 1. Whole body clearance of ^{123}I-SAP in amyloidotic (■) and control (□) mice. Each point represents the mean ± SD of three animals. (*A*) Human SAP; the difference between groups was significant at 4 h ($P < 0.01$) and at 24 h ($P < 0.001$). (*B*) Mouse SAP; the difference between groups was significant at 5 h ($P < 0.05$) and at 24 h ($P < 0.01$).

organ was then snap frozen for histological examination, the remainder stored at −20°C, and subsequently assayed for the presence of AA protein by RIA.

Statistical Analysis. Histological and RIA estimates of amyloid in each organ were tested for significant correlation using Spearman's rank correlation coefficient (R). Correlations between localization of SAP and these estimates of amyloid quantity were also tested in the same way. Statistical significance of differences in whole body clearance and organ distribution of labeled SAP was sought using Student's *t* tests.

Results

Gamma Camera Imaging after Injection of ^{123}I-Human SAP. After intravenous injection of ^{123}I-human SAP into normal, control mice, gamma camera images showed the presence of activity compatible with its distribution in the blood pool. There was rapid whole body clearance so that only 65% of injected counts remained after 4 h and 25% or less at 24 h (Fig. 1*A*). Over this period there was some uptake of activity into the thyroid glands, which were not blocked beforehand, and minor retention of iodinated material in the upper abdomen and the bladders of some animals. Control mice killed at 24 h had a significant proportion of the total body counts in their stomachs, presumably as a result of swallowing excreted material during grooming, combined with gastrointestinal stasis due to repeated ether anesthesia. There was no evidence of any significant localization of activity elsewhere in the control animals (Fig. 2).

In contrast, mice that had received repeated casein injections, and that all had amyloidosis, yielded distinctly different images within 30 min of injection of ^{123}I-human SAP. There was major, specific localization of activity in their spleens and livers (Fig. 2), no localization elsewhere, and significant whole body retention of counts compared with the controls (Fig. 1*A*). These results were confirmed by the counts detected in the individual organs removed 24 h after injection (Fig. 3), corresponding exactly with the presence of amyloid in histological sections. None of the control animals contained any amyloid, whereas the casein-injected mice all had similar, massive amyloid deposits in their spleens and definite widespread, although less extensive, deposits in their livers. There were

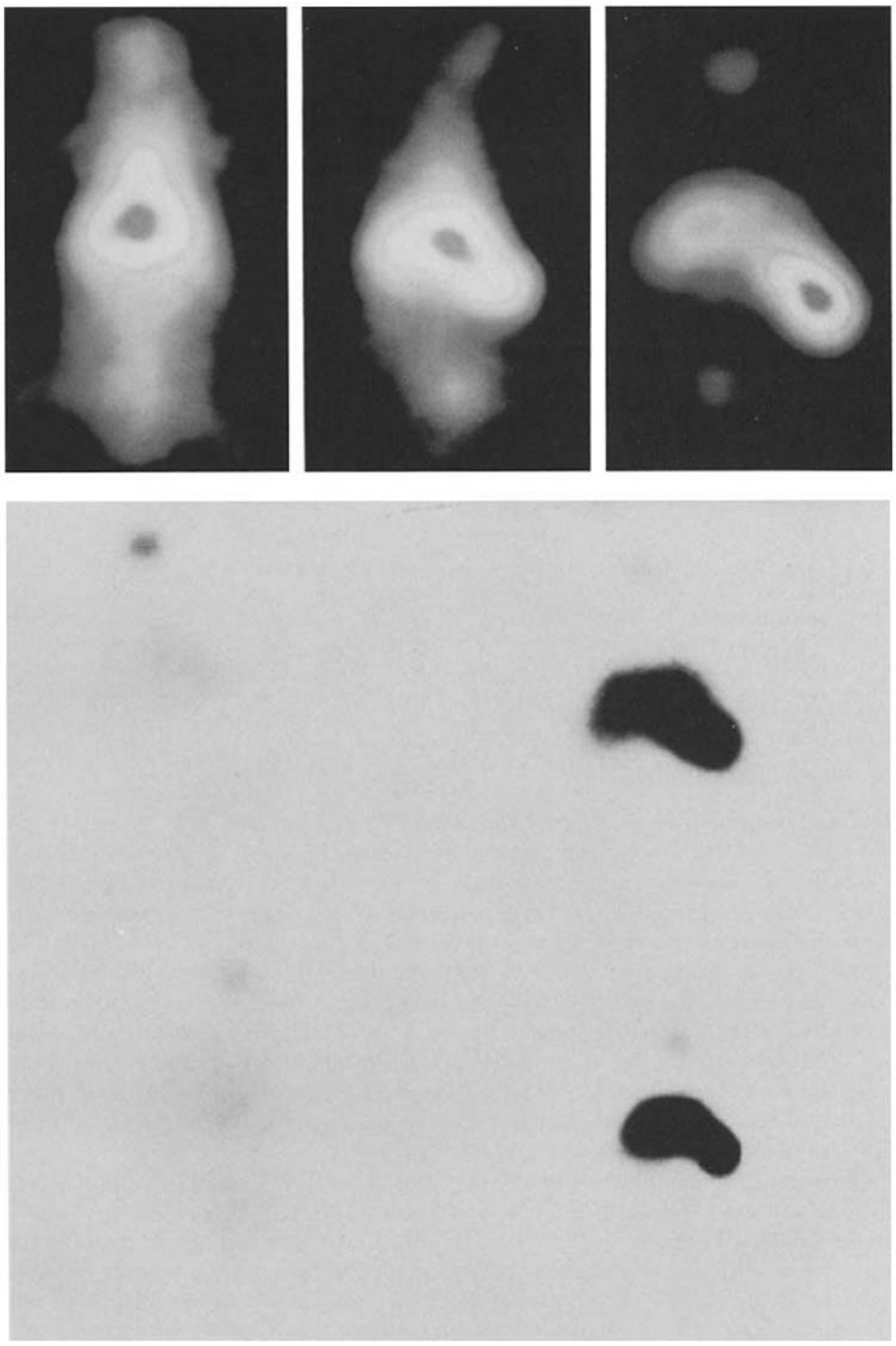

FIGURE 2. Scintigrams of mice after intravenous injection of ^{123}I-human SAP. (*Top*) Serial images of a single casein-induced amyloidotic animal in a ventral projection taken at time 0 after injection (*left*), 0.5 h (*center*), and 19 h (*right*), showing initial distribution in the blood pool followed by rapid sequestration and retention in the amyloid-laden liver and spleen with complete clearance elsewhere. (*Bottom*) Composite image of four mice 24 h after injection. Controls on left top and bottom; casein-induced amyloid mice right top and bottom. The latter show massive localization to the liver and spleen with traces of activity in the thyroid; the controls show thyroid activity only, with even less elsewhere.

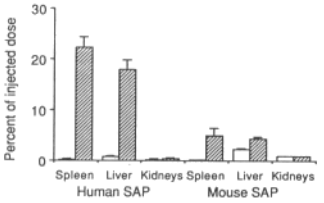

FIGURE 3. Organ localization of ^{125}I-SAP in casein-induced amyloidotic (▨) and control (□) mice. Each bar represents mean ± SD of three mice.

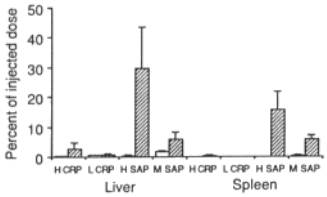

FIGURE 4. Organ localization of ^{125}I-pentraxins in AEF-induced amyloidotic (▨) and control (□) mice. Each bar represents mean ± SD of five mice. *H*, human; *L*, *Limulus*; *M*, mouse.

only minute traces of amyloid in the kidneys of some of the test mice and none was found elsewhere.

Gamma Camera Imaging after Injection of ^{123}I-Mouse SAP. Injection of labeled mouse SAP gave results similar to those seen with human SAP except that the proportion of the dose localized to amyloidotic organs was smaller (Fig. 3). Furthermore, there was more rapid clearance of organ-localized activity than occurred with labeled human SAP. Finally, whole body retention of ^{123}I-mouse SAP was more prolonged than that of human SAP and there was little difference between amyloid and control mice (Fig. 1*B*), as we have reported previously for ^{125}I-mouse SAP (24). As a result, the production of gamma camera images at 24 h took longer, the blood background was higher, and contrast was slightly less good.

Localization of ^{125}I-Pentraxins in AEF-induced Amyloid. All the animals that received AEF and silver nitrate became amyloidotic, but there was generally much less amyloid present than in casein-injected animals as well as much greater variation between individuals. The quantity of amyloid present in each organ at the end of each experiment was estimated independently by histology and by RIA for tissue AA. The results correlated in general, though by no means absolutely: livers, Spearman's rank correlation coefficient $r = 0.421$, $n = 20$, $p < 0.05$; spleens, $r = 0.478$, $n = 20$, $p < 0.05$. These results reflect the considerable difficulty in obtaining reliable, quantitative estimates of the amount of amyloid in affected tissues.

However, the specificity of deposition of injected SAP in amyloid deposits was confirmed using ^{125}I-trace-labeled human and mouse SAP, compared with human and *Limulus* CRP as controls. The CRP molecules closely resemble their SAP counterparts in terms of structure but differ with respect to their calcium-dependent ligand-binding specificity. Both human and mouse ^{125}I-SAP showed highly significant specific uptake into amyloidotic compared with normal, control livers and spleens, and the proportions of the injected doses that localized were similar to those seen in the imaging experiments (Fig. 4). Labeled human CRP showed significantly greater uptake into amyloidotic than normal organs, but the quantities involved were extremely small; e.g., the amount in the spleen was

TABLE I

Quantitation of Tissue Amyloid Deposits

^{125}I-Protein	Pretreatment	Liver			Spleen		
		Histology	RIA	^{125}I-Protein	Histology	RIA	^{125}I-Protein
Human SAP	AEF/$AgNO_3$	1.5 (0.5–5.0)	4.5 (1–19)	21.6 ± 9.4	4.3 (2.5–10)	35 (7.1–57)	227.2 ± 66.0
Human SAP	—	0	0	0.4 ± 0.1	0	0	0.8 ± 0.3
Mouse SAP	AEF/$AgNO_3$	5.0 (1–10)	12.0 (4.7–55)	4.6 ± 1.5	22.0 (10–40)	28 (22–188)	59.1 ± 29.4
Mouse SAP	—	0	0	1.8 ± 0.2	0	0	5.9 ± 8.4
Human CRP	AEF/$AgNO_3$	3.9 (0.5–5)	17.0 (5–23)	1.9 ± 1.2	10.0 (4–20)	65 (20–402)	6.1 ± 5.9
Human CRP	—	0	0	0.3 ± 0.1	0	0	0.7 ± 0.5
Limulus CRP	AEF/$AgNO_3$	2.7 (0.5–5)	35.0 (19–50)	0.6 ± 0.0	5.0 (1–25)	60 (20–105)	0.6 ± 0.2
Limulus CRP	—	0	0	0.5 ± 0.1	0	0	0.9 ± 0.2

Histological score (median [range]) expressed as percent area of microscope fields scanned containing green birefringent amyloid; RIA results are AA content/g of tissue (median [range]) expressed as percent of standard AA-rich spleen (see Materials and Methods); ^{125}I-protein localized at 24 h expressed as percent of injected dose/g of liver or spleen (mean ± SD). Five mice in each group.

~10% of that of mouse SAP and 2.5% of human SAP. *Limulus* CRP did not show any such localization at all.

Despite the qualitative specificity of deposition of labeled SAP in amyloidotic organs (Fig. 4, Table I), there was relatively poor correlation between the amount of label present and the estimates of amyloid either by histology or RIA. Thus, only the following correlation coefficients were significant ($P < 0.05$): RIA vs. human SAP and histology vs. human SAP in the liver, and histology vs. mouse SAP in the liver and histology vs. mouse SAP in the spleen. However, if the control mice, in which there was no amyloid by either test and no SAP localization, were included, the correlations became highly significant ($P < 0.001$) in all comparisons.

The lack of precise correlation between counts localized and estimates of "severity" of amyloid in the amyloidotic animals may reflect, once again, the difficulty in estimating amounts of amyloid present, but could also result from variation between animals in the availability, in its broadest sense, of amyloid for binding by SAP. There was also some evidence of differences between amyloid deposits in different organs with respect to their capacity to take up labeled SAP. Thus, much more labeled SAP localized per gram of tissue in the spleen than in the liver (Table I) and the ratio of these values generally exceeded the ratio of estimated quantities of amyloid in the respective organs.

Discussion

The present results demonstrate that ^{123}I-labeled SAP can be used to provide specific high resolution gamma camera images of tissue amyloid deposits in vivo. A remarkable proportion of the injected dose of SAP localizes in organs containing amyloid. After 24 h, this was ~10% with mouse SAP and an extraordinary 40% with human SAP, after injection of 100 μg of protein in each case. These values compare with typical results of <1% of the injected dose of monoclonal antitumor antibodies that localize specifically to neoplastic lesions in man or animals (25). Human SAP that had localized to amyloid deposits was retained there with little loss between 4 and 24 h after injection, in contrast to its rapid and extensive whole body clearance in control mice. The whole body clearance of mouse SAP was slower than that of human SAP but was similar in amyloidotic

and control mice. Together with the smaller proportion of the injected dose that localized to amyloid, this suggests that mouse SAP has a lower affinity for binding to isologous AA fibrils than does the heterologous protein, human SAP. This is a novel and interesting finding that merits further investigation especially in the light of the high resolution three-dimensional structure of human SAP which will soon become available (26, 27). However, in purely operational terms, it means that human SAP is a better targeting agent for mouse AA amyloid than is mouse SAP.

Of the control proteins studied, *Limulus* CRP did not localize at all to amyloid deposits, while human CRP showed significant localization but only at a trace level. This is not surprising since human CRP and SAP show a slight overlap in ligand-binding specificity (23, 28, 29), and we have reported previously that this may extend to the ligand(s) on human amyloid since CRP showed weak binding to two of three different AL fibril preparations (21). In further experiments, not shown here, we have investigated the in vivo localization to mouse AA amyloid deposits of the two distinct bovine pentraxins that we recently characterized for the first time (30). Both localized to amyloid, one as well as human SAP, and the other less well, but still appreciably better than mouse SAP. There is thus scope for further study of heterologous pentraxins in this experimental system and possibly also in a clinical context in the future.

In a previous, preliminary report, we established that low resolution, but nonetheless, diagnostic images of murine amyloid could be obtained in vivo after injection of ^{131}I–human SAP (31). The superior, high resolution results shown here reflect the excellent properties of ^{123}I with its high energy (0.159 MeV) γ emission and high photon yield in the gamma camera. Other advantages, particularly for prospective clinical applications, are the low β emission, the short half-life (13 h), and the ease of efficient protein labeling. Unfortunately, however, ^{123}I is available only from a limited number of centers. More widely available isotopes suitable for scintigraphy are ^{99m}Tc and ^{111}In, but the former is not readily conjugated with native proteins, and after injection of proteins labeled with ^{111}In there is marked nonspecific uptake of isotope in the liver. This is well known in man (32) and we have encountered the same problem in mice with ^{111}In-SAP labeled by the diethylenetriaminepentaacetic acid method (unpublished observations).

The qualitative specificity of localization of injected SAP to amyloid has been well established here, but a major question remaining is whether the quantity of SAP that localizes reflects the absolute amount of amyloid present. This question is difficult to answer precisely because of the problems involved in accurate estimation of the amyloid content of tissues. Histological examination of Congo red–stained sections cannot reasonably sample more than a very small proportion of the liver or spleen, and even then light microscopic evaluation of green birefringent areas may not reflect quantitatively the absolute amount of amyloid fibril deposits. Nevertheless, in mice with casein-induced amyloid, which all had similar and extensive Congophilic, green birefringent deposits in their spleens and livers, there was very little variation in the proportion of the injected dose of labeled SAP that localized. On the other hand, in the AEF-induced model there was much less amyloid in the tissues assessed histologically, and much

greater variation between mice than in the casein-induced model. While in these mice the absolute specificity of SAP for amyloidotic organs was retained, there were only imperfect correlations between labeled SAP localized and histological score.

Our RIA for tissue AA was intended to overcome the sampling and interpretation problems of histology. It was highly specific and gave no false-positive results but showed only partial correlation with Congo red staining and with quantity of SAP localized. Given the complexity of amyloid deposits, including fibrils of different ages, glycosaminoglycans, and SAP, there is no a priori reason to expect that there should necessarily be a close correlation between Congo red birefringence (the precise molecular basis of which is not known), AA antigen content, and SAP deposition. Indeed we observed here that appreciably more SAP seemed to localize in the spleen than in the liver in association with apparently similar quantities of amyloid.

It is possible, therefore, that these variable correlations seen with AEF-induced amyloid and trace-^{125}I-labeled SAP were due to inherent features of amyloid pathology, rather than merely the operational problems of amyloid quantitation. For example, the ligand on amyloid fibrils to which SAP binds has not yet been biochemically identified; it is not known whether it is exposed on all fibrils at all times during the pathogenesis of fibril deposition, nor whether competitive ligands exist either in body fluids or on other tissue components. Another potentially important variable is local vascular permeability, which may determine when, where, and how much SAP (M_r 254,000 [reference 27]) leaves the circulation to enter amyloidotic tissue. It will be important, therefore, to determine whether or not SAP localizes with the same kinetics and to the same extent in relation to similar amounts of amyloid fibrils in different tissue sites and at different stages of the disease. Regardless of the outcome of such studies, we have here demonstrated for the first time a noninvasive procedure for specific in vivo imaging of amyloid that is potentially applicable in man, and studies in patients with various forms of amyloid are currently in progress. Diagnostic imaging methods based on the presence of abundant calcium in amyloid deposits (33) have previously been used clinically, but they are not specific and have limited sensitivity (34, 35). Imaging of AA amyloid in mice has been achieved with radiolabeled monoclonal anti-AA antibodies (36), but this is clearly not an approach that could be generally applicable in man, given the diversity of amyloid fibril proteins and the problems posed by injection of heterologous Igs.

Availability of a repeatable in vivo diagnostic procedure should increase knowledge of the natural history of amyloid and may ultimately permit early diagnosis in groups at risk, such as chronic haemodialysis, rheumatoid arthritis, and juvenile chronic arthritis patients. Another possible application is in Alzheimer's disease and senile dementia of Alzheimer type, presently diagnoses of exclusion, but in which cerebrovascular amyloid containing SAP is almost universally present (7, 37–40). Finally, the present successful demonstration of specific localization to amyloid of intravenously injected radiolabeled SAP suggests that SAP could also be used as a targeting vehicle to transport to amyloid deposits substances with potentially beneficial therapeutic effects. Experiments to test this concept are in progress.

Summary

Highly specific, high-resolution scintigraphic images of amyloid-laden organs in mice with experimentally induced amyloid A protein (AA) amyloidosis were obtained after intravenous injection of ^{123}I-labeled serum amyloid P component (SAP). Interestingly, a much higher proportion (up to 40%) of the injected dose of heterologous human SAP localized to amyloid and was retained there than was the case with isologous mouse SAP, indicating that human SAP binds more avidly to mouse AA fibrils than does mouse SAP. Specificity of SAP localization was established by the failure of the related proteins, human C-reactive protein and *Limulus* C-reactive protein, to deposit significantly in amyloid and by the absence of human SAP deposition in nonamyloidotic organs. However, only partial correlations were observed between the quantity of SAP localized and two independent estimates, histology and RIA for AA of the amount of amyloid in particular organs. It is not clear which of the three methods used reflects better the extent or clinical significance of the amyloid deposits but in vivo localization of radiolabeled SAP, detectable and quantifiable by gamma camera imaging, is apparently extremely sensitive. These findings establish the use of labeled SAP as a noninvasive in vivo diagnostic probe in experimental amyloidosis, potentially capable of revealing the natural history of the condition, and suggest that it may also be applicable generally as a specific targeting agent for diagnostic and even therapeutic purposes in clinical amyloidosis.

We thank Professor J. P. Lavender for helpful discussions of the study and the manuscript, and Beth Sontrop for expert preparation of the manuscript.

Received for publication 26 October 1987.

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