Virchows Arch (2000) 436:439–448

ORIGINAL ARTICLE

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Highly sensitive diagnosis of amyloid and various amyloid syndromes using Congo red fluorescence

Received: 29 September 1999 / Accepted: 2 December 1999

Abstract In order to find how best to diagnose amyloid deposits as early as possible, the sensitivity of three different methods that can be applied to the diagnosis of amyloid in tissue sections have been compared: the Congo red staining method (CR), the combination of CR and immunocytochemistry (CRIC) and Congo red fluorescence (CRF). Tissue blocks were available from 25 patients, including 11 with immunohistochemically distinct and 3 with chemically undefined amyloid diseases. The results revealed (a) that CRF is more sensitive than either CR or CRIC, as shown qualitatively and quantitatively, (b) that CRF can therefore be utilized to track down even minute amyloid deposits, which can be missed by the other two methods; (c) that the specificity of CRF and CRIC is secured on double-stained sections by the demonstration of green birefringence (GB) of the CRF-marked and IC-marked areas; (d) that CRF can be performed on the spot by just changing the light source; and (e) that CRF is *not* hampered by the congruent IC chromogen overlay, which ensures the specific classification of the amyloid deposits as applied to different amyloid classes. In conclusion, CRF was demonstrated to be the most sensitive method for direct diagnosis of amyloid in tissue sections. This method can, therefore, allow the earliest diagnosis and classification of amyloid, which is a good basis for an amyloid class-specific therapy while organ damage is still minimal.

Key words Amyloid · Classification · Congo red fluorescence · Early diagnosis · Immunohistochemistry

This work was presented in part at the 13th Amyloidforum, 29 May 1999, in Jena/Germany during the 83rd Annual Conference of the German Society of Pathology

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Introduction

Amyloidosis comprises a large group of different diseases, which are characterized by symptoms caused by the deposition of amyloid in vital organs. These diseases are usually progressive, and most amyloid syndromes are fatal [4, 12, 20]. Nevertheless, some of these diseases can now be treated successfully [1, 6, 16, 21, 38].

Before the therapy of a suspected amyloid disease can be planned, both the presence of amyloid and its chemical origin must first be known [4, 12, 20]. To this end, a reliable, sensitive and specific procedure for diagnosing amyloid is required as a first step.

Unfortunately, the Congo red staining method currently applied routinely [32] has been shown to be unreliable in some cases [15, 29, 30], in particular when only small amounts of amyloid are present, and radioactive imaging has not been applied to early amyloidosis controlled by biopsies [15].

To increase the sensitivity of the bioptic diagnosis of amyloid, Congo red staining (CR) and immunocytochemistry (IC) have been applied together in various combinations (CRIC). It has been shown that CRIC is more sensitive then CR alone [29]. It has also been demonstrated that this increased sensitivity can verify the correct diagnosis several years earlier. Furthermore, the estimated benefits of an early diagnosis for the patient, in terms of being spared further invasive diagnostic measures and of receiving a timely and appropriate therapy, have also been reported [30].

The CRIC technique used to bring forward the diagnosis of amyloid and to improve the classification of it was further refined in the present study by utilizing Congo red fluorescence (CRF); this procedure increases the recognition of tissue-bound Congo red by virtue of its property as a fluorochrome [31].

In this study, CRF was applied to a panel of bioptic and autoptic tissues affected by various chemically different amyloid diseases. It was possible to demonstrate superior sensitivity of CRF in terms of achieving the correct diagnosis, especially when only minute amounts of

amyloid are present, and the feasibility of utilizing CRF to increase the specificity of the immunohistochemical classification of various amyloid syndromes.

Materials and methods

Tissues and diagnosis of amyloid

Formalin-fixed (4% buffered formaldehyde) tissue sections approximately 4–6 µm thick were sent in for consultation from various institutions. The clinical data of patients examined and the senders of the tissue specimens are listed in Table 1. Amyloid was diagnosed using the stringent, alkaline, alcoholic Congo red (Aldrich Chemical Co., Milwaukee, Wis.) staining method of Puchtler et al. [32]. The Congo red (CR)-stained sections were weakly counterstained with Mayer's acid hemalum (E. Merck, Darmstadt, Germany), embedded in Kaiser's glycerin jelly (E. Merck) and examined in bright and polarized light using a Zeiss standard microscope with built-in polarization equipment. A high-quality microscope, and in particular the use of tension-free optics and high-quality polarization filters, is indispensable. A rotating slide table is also required for polarization microscopy.

The characteristic green birefringent polarization color (GB) was taken as proof of the presence of amyloid when it was sited within the tissue structure [4, 7, 12, 13, 22].

It is clear from the literature that whenever GB is negative a diagnosis of amyloid cannot be made, regardless of the reasons, including poor equipment or inappropriate procedures of staining and evaluation. For some of the amyloids the N-terminal or the full amino acid sequence of the amyloid fibril proteins was determined (for references see Table 1). Monoclonal and polyclonal antibodies against selected, purified, amyloid fibril proteins were available (see "Immunohistochemistry").

Congo red fluorescence and photography

The Congo red fluorescence (CRF) [31] was induced on CR-stained paraffin tissue sections using a high-pressure mercury burner (HBO 200, W/4) as part of a Zeiss standard microscope with photographic equipment. A filter set for detecting fluorescein isothiocyanate (FITC), with an absorption maximum of 495 nm (blue) and an emission maximum of 525 nm (green), and tetramethylrhodamine isothiocyanate (TRITC), with an absorption maximum of 555 nm (green) and an emission maximum of 580 nm (red), was used. Photography was performed either in bright, polarized or in ultraviolet (UV) light on color reversal films for tungsten light using Fujichrome 64T from Fuji and Ektachrome 64T from Kodak.

Immunohistochemistry

Amyloid deposits in formalin-fixed and paraffin-embedded tissue sections were immunostained using the unlabelled immunoperoxidase technique of Sternberger as described in detail previously [23, 25, 39] with Na-perborate (E. Merck) as a substrate and 3-amino-9-ethylcarbazol (AEC, grade II, Sigma, St. Louis, Mo.) as a chromogen. Monoclonal and polyclonal antibodies were prepared and characterized as previously described [23, 25, 39]. Each of the antibodies used was shown to react largely with all amyloid deposits of the same class from different patients, while most were less reactive or unreactive with other amyloid classes. Monoclonal antibodies directed against cytokeratin (code: MNF116) were from Dako, Hamburg, Germany. Anti-Aβ [37], anti-ANP [28], anti-IAPP [33], anti-FibAα (in progress), and anti-prolactin antibodies (in progress) were prepared by us in rabbits. Anti-cystatin-c was a gift of Professor Machleidt, Munich. The sections were weakly counterstained and embedded as described above.

Diagnostic procedures

CR-stained [32] sections were microscopically evaluated in bright and polarized light (Fig. 5). In cases where GB was detected in a CR-binding area, the diagnosis of amyloid was made [4, 12, 13]. CRF was applied when no amyloid was found. CRF-illuminated areas were then examined in polarized light for the presence of GB (objectives 16.0 and 40) with maximal light.

When no amyloid was detected by CRF together with GB, a series of 10 or more additional sections were stained and examined in the same manner to eliminate sampling error [29]. In cases where only minute amounts of amyloid were revealed in some of the sections by CRF and GB, the location of each amyloid spot was documented by sketching. After immunohistochemical staining of the same sections using our set of amyloid fibril protein antibodies (see above), the sketched areas were then evaluated in bright, polarized and UV light. Since the strongest and most consistent immunohistochemical reaction identified the amyloid class [23, 25], all amyloid spots were carefully examined for the IC-marking. The amyloid classes identified in this report are listed in Table 1.

Comparison of methods

In order to compare the sensitivity of the three methods, CR, CRIC, and CRF, six paraffin blocks with rectal and renal biopsies from patients with the clinical conditions shown in Table 1 were selected for this quantitative study. One biopsy (Table 1; patient 2, first biopsy) had no amyloid and served as a negative control. Two further biopsies (patient 2, second biopsy; patient 3, second biopsy) with larger amounts of amyloid in all sections served as positive controls. Four additional biopsies (patient 3, first and third biopsies; patients 4 and 5) had various amounts of amyloid or no amyloid at all in individual sections (sampling error [29]). These four biopsies from previous work [29] have been used here as an indicator for the sensitivity of the three methods CR, CRIC, and CRF. The tissue blocks were sectioned up to the last bit of tissue, and 57 (negative control), 128 (two positive controls) and 211 (various very small amounts of amyloid in four tissue blocks) sections were retrieved from these biopsies.

All 396 sections were stained with CR [32] and microscopically evaluated for the presence of amyloid in a blind fashion. To ensure that examinations were done a blind fashion, each slide was handed to the observer by a technician, who also recorded the evaluation. The observer was unaware of any of the patient's data.

The first readings were performed using CR only. After the data were documented, CRIC was applied to adjacent and/or to the same sections. The last examination was performed with CRF. The readings were performed time and again until all positive sections were recognized using a given technique and the readings became stable and consistent. This stage was reached when at least two consecutive readings were virtually identical. When a new result appeared in one section of a given biopsy the remaining parallel sections of the same biopsy were reexamined to find out whether or not this new finding had a bearing on the results of the other biopsies. Interobserver variability was not examined, but it should be checked on in the future and various expert centers should be included.

Subsequently, all the sections were immunohistochemically stained using our monoclonal anti-AA (mc1) antibody [23]. The sections were then evaluated for the presence of amyloid by searching for IC-marked areas that revealed GB, also in a blind fashion. The third examination included CRF. Amyloid was diagnosed by the identification of either CRIC- or CRF-marked areas that were congruent with areas showing GB. A direct comparison of the same area by bright, polarized and fluorescent light can be performed on the spot, simply by switching the light source.

Examination of various amyloid diseases using CRF

CRF was applied to a series of tissue sections containing chemically different amyloids (Table 1) to examine whether the different

Table 1 Comparison of Congo red staining and Congo red fluorescence in 25 patients representing various amyloid classes. Clinical data and source of tissue. Qualitative data are derived from tissue of patient 1 and documented in Figs. 1–3, while quantitative data from patient 2–5 are documented in Fig. 4 (*A* autopsy *B* biopsy, *F/M* female/male, *AA* amyloid A, *Aλ* amyloid of immunoglobulin λ-light chain origin, *Aκ* amyloid of immunoglobulin κ-light chain origin, *TTR* transthyretin, *ATTR* amyloid of transthyretin origin, *S* sporadic [*ATTRS* systemic senile cardiovascular amyloidosis], *F* familial [ATTR*F* familial amyloid polyneuropathy], patient 13 had a TTR mutation [Val 20 Ile], *Aβ$_2$M* amyloid of beta$_2$-microglobulin origin, *"AK"* amyloid most probably of cytokeratin origin [14], but not formally proven, *AANF* amyloid of atrial natriuretic protein origin, *Aβ* amyloid of β-protein origin, *ACys* cerebral amyloid angiopathy of cystatin C origin, *AFib* amyloid of fibrinogen Aα origin, *APro* amyloid of prolactin origin, *AIAPP* amyloid of islet amyloid polypeptide origin, *N.R.* not reported, *Ref.* references referring to scientific reports giving further details on the chemistry and/or immunohistochemistry of the amyloid fibril protein of the respective patient, *CR* Congo red (staining), *CRIC* Congo red and immunohistochemistry, *CRF* Congo red fluorescence)

Patient	Code[a]	Age	Sex	Clinical syndrome	Organ	CR[d]	CRIC[f] amyloid class	CRF[d]	Ref.	Sender
1	286	25	F	Muckle-Wells syndrome	A: kidney	+++	AA	++++	[24]	Prof. Dr. K.L. Heilmann,[j] Landshut
2	1519, 1	8	M	Juvenile rheumatoid arthritis	B: kidney	0	0	0	[29]	
	1519, 2	9			B: rectum	+++	AA	++++		
3	1416, 1	12	M	Systemic vasculitis	B: kidney	+++[e]	AA	++++	[29]	Dr. H. Michels,[k] Garmisch/Neckargemünd
	1416, 2	13			B: kidney	+++	AA	++++		Prof. Dr. A. Bohle,[j,k] Tübingen
	1416, 3	15			B: kidney	+++[e]	AA	++++		Prof. Dr. V. Gärtner,[k] Tübingen
4	1150, 9	12	F	Familial Mediterranean fever	B: rectum	+++[e]	AA	++++	[29, 30]	
5	1150, 15	8	F	Familial Mediterranean fever	B: rectum	+++[e]	AA	++++	[29]	
6	2336	51	M	κ-LCDD[b] (Aκ-amyloid)	A: myocardium	0 (++)	κ-deposits (Aκ)	0 (+++)		Prof. Dr. J. Caselitz, Hamburg
7	546	64	F	λBJ-plasmocytoma	A: kidney	++	Aλ	++++	[10]	Prof. Dr. J. Saal, Tübingen/Kiel
8	339	36	F	IgD, λ multiple myeloma	A: kidney	++	Aλ	++++		Prof. Dr. K.L. Heilmann,[j] Landshut
9	6	53	F	IgG, κ multiple myeloma	A: tongue	+++	Aκ	++++	[9]	Dr. Fuhrmann, Stuttgart
10	2695	57	F	Nodular Aκ-amyloidosis	B: skeletal muscle	+++	Aκ[g]	++++		Dr. E. van Almsick, Berlin
11	658, 1	81	M	N.R.	A: atrium	++	ATTRS	++++	[17]	Dr. H. Hüsselmann, Hamburg
12	35	88	M	Myocardiopathy	A: heart	+++	ATTRS	++++	[36]	Prof. Dr. W. Selberg, Hamburg
13	2099	64	M	Familial hypertrophic cardiomyopathy	B: rectum	++	ATTRF	++++	[18]	Dr. G. Ricken, Freiburg
14	720	66	M	Renal failure, hemodialysis	A: synovia	+++	Aβ$_2$M	++++	[27]	Prof. Dr. H. Lobeck, Berlin
15	1833	76	M	Lichen amyloidosus	B: skin	(+)	"AK"[h]	++		Dr. W. Undeutsch, Wangen
16	2831	60	M	Notalgia paresthetica	B: skin	+	"AK"[h]	+++		Prof. Dr. C. Schmoeckel, Munich
17	1899	62	M	Cardiomyopathy	Atrium of explanted heart	++	AANF	+++	[28]	Dr. A. Schnabel, Heidelberg
18	1445	61	M	Alzheimer's disease	A: cerebral cortex	++	Aβ	++++		Prof. E. Braak, Frankfurt
19	818	37	M	Hereditary Icelandic apoplexy	A: cerebral cortex	+++	ACys	++++	[11]	Prof. Dr. O. Jensson, Reykjavik
20	1970	N.R.	N.R.	Familial amyloid polyneuropathy	A: kidney	+++	AFib	++++	[2]	Prof. Dr. M. Benson, Indianapolis, Ind.
21	2830	30	F	Prolactinoma	B: pituitary gland	+++	APro[g]	++++		Prof. Dr. R. Schröder, Cologne
22	430	75	F	Aλ-amyloidosis	A: pancreas	+++	Aλ	++++	[33]	Prof. Dr. U. Gross, Berlin
				Cardiomyopathy[c]		+++	AIAPP	++++		
23	2723, 7	62	M	N.R.; interstitial pituitary amyloid	A: pituitary gland	++	Unknown[i]	+++		Prof. Dr. K.M. Müller, Bochum
24	2512	90	M	Metastatic colon Ca, "portal amyloid"	A: ilium	++	Unknown	++++	[34]	Prof. Dr. W. Nathrath, Munich
25	947,1	81	M	N.R.; "elastic amyloid"	B: temporal artery	++	Unknown	+++	[26]	Prof. Dr. T. Muckle, Hamilton, Ont.

[a] Internal code
[b] Double protein thesaurosis, with identification of κ-LCDD and Aκ-amyloid (the latter in brackets)
[c] Double amyloid with Aλ in vessels and interstitium, and AIAPP in the islets of Langerhans
[d] Intensity of staining, not amount of amyloid (grades: ++++, very intensive [see Figs. 1c, 2c]; +++, intensive; ++, strong; +, weak but definitive; (+), very weak but definitely showing GB or CRF; 0, no GB and no CRF
[e] Not all sections contained amyloid; the proportion detected is dependent on the method used (Fig. 4)
[f] Immunohistochemical classification (see "Methods")
[g] In progress
[h] Keratin nature not established
[i] Unreactive with the antibodies used in this report, but reactive with an antibody directed against the amyloid-P component
[j] Sadly Professor Heilmann and Professor Bohle have died since this work was begun
[k] Dr. Michels (physician) and Professor Gärtner and Professor Bohle (pathologists) all provided samples taken from patients 2–5

amyloids revealed the same intense fluorescent illumination and whether or not CRF would be useful in detecting amyloid after the immunohistochemical classification of chemically different amyloid diseases.

Ethical standards

This study was approved by the local ethics committee. The living persons mentioned gave their informed consent.

Results

Comparison of CR and GB

Amyloid in CR-stained fixed paraffin sections revealed the typical pale pinkish to pink-reddish coloration in bright light (Fig. 1a). This staining can become more intense with increased thickness of the sections. When the section was more than approx. 15 µm, however, thick amyloid appeared more yellowish, and even orange in certain parts under polarized light, as has been shown before [25, 42]. In addition, sections less than 2 µm thick showed less intensive staining (not documented). Moreover, amyloid deposits of different patients differed in their intensity of CR staining even when the sections appeared to be similar in thickness, suggesting that amyloid has different binding capacities for CR in different patients, as has been seen before [32, 40]. As examples, the amyloid in lichen amyloidosus seems to take up CR less intensely than AA, ATTR, most AL amyloids, and other amyloids (Table 1).

In polarized light, the same sections display GB, which identifies amyloid after stringent staining [4, 12, 13, 25, 29]. However, in contrast to the situation when the diagnosis is made in bright light, only some areas of the amyloid mass are immediately identifiable as amyloid by GB at first glance. The other parts of amyloid identified by CR binding lack the GB in the same view, as illustrated in Figs. 1b, 2b, and 3b. When the section is rotated, however, the formerly GB-illuminated areas lose their GB, while other formerly black areas reveal GB. When the slide was rotated further this step-by-step maneuver made it possible to confirm that all CR-binding material represented amyloid with GB. Thus, the congruence between CR staining and GB is only obvious in all CR-stained areas if the slide table is rotated. The amyloid-containing areas not revealing GB will be referred to in this report as being in the "polarization shadow."

Comparison of CR with IC

IC-marked areas and CR-binding areas (that revealed GB) were *congruent* and *visible at first glance* in most parts (Figs. 2a, b, 3a, b), in contrast to the GB label, which is rotation dependent (Figs. 2b, 3b). In addition, IC is shown qualitatively to be far more sensitive than the CR staining alone (Figs. 1a, 2a), as has been demonstrated previously [29]. When the sections were pre-stained with CR and subsequently with IC, the polarization color seemed more yellowish or yellowish green when overlaid with the chromogen AEC (Figs. 2b, 3b). In addition, one also has the impression that the chromogen overlay somewhat impedes the results obtained by polarized light. This applies more to the amyloid of a lesser order, as exemplified in glomerular amyloid where only approximately 20% is visible by GB at first glance (Figs. 1b, 3b). However, in vascular amyloid approximately half the amyloid is revealed by GB, as shown in Fig. 2b, due to the better alignment of the amyloid bundles along the smooth muscular cells.

Qualitative comparison of CRF with CR and CRIC

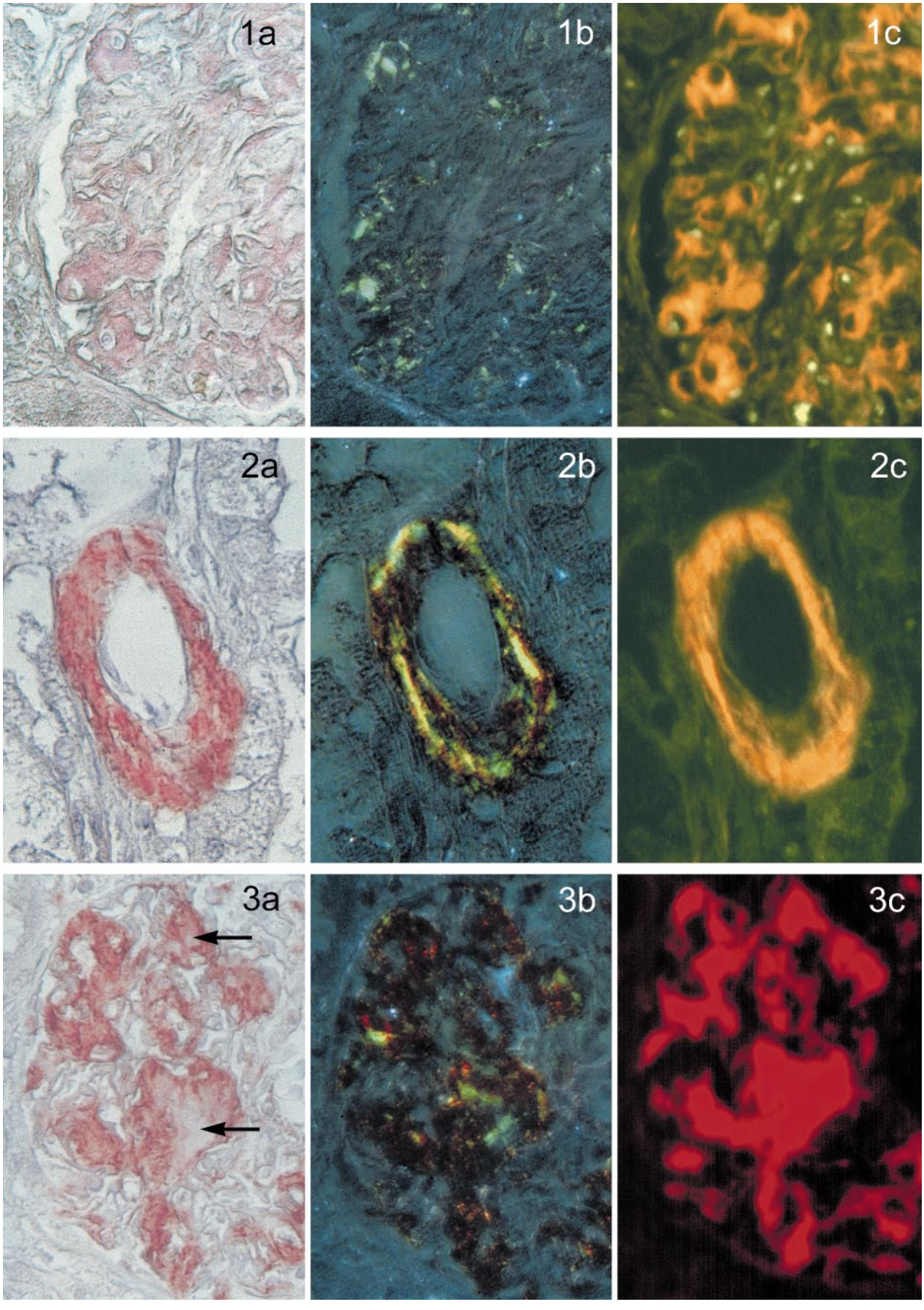
When UV light was used, the CR-stained amyloid was illuminated in bright red with a red background of lesser intensity visible with the TRITC filter set (Fig. 3c). Using the FITC filter set, however, amyloid gleamed bright yellow-orange with a dark green background fluorescence of the nonamyloid tissue in most parts (Figs. 1c, 2c). Since the distinction of amyloid with the latter filter set was more differential, this filter was used throughout this study.

While the illumination using CRF was absolutely congruent with the CR staining, it was much more intensively shining in UV than CR in bright light (Fig. 1a, c). CRF also revealed congruency with IC staining, in contrast to GB, as shown in Fig. 2a, c. A comparison of IC- and CRF-marked areas reveals that CRF is even brighter than the areas of IC staining (Fig. 2a, c). Although the areas containing amyloid are all marked likewise in a congruent mode by IC and CRF, however, the CRF seems to be stained not only more intensively but also more uniformly than IC. The variable IC staining of different amyloid areas is clearly visible and indexed by ar-

Fig. 1a–c Visualization of amyloid deposits in autoptic formalin-fixed and paraffin-embedded renal tissue from a patient with Muckle-Wells syndrome (patient 1 in Table 1). Comparison of CR staining of amyloid showing the same area in **a** bright, **b** polarized and **c** UV light. All areas stained in pink as seen in **a** are congruent and brightly orange fluorescent at first glance in **c**, while the amyloid-free background is mostly dark green (**c**). In contrast, GB does not illuminate all amyloid due to the "polarization shadow". Light hemalum counterstaining, ×380 ▶

Fig. 2a–c Visualization of amyloid deposits in tissues from the same patient as in Fig. 1. Renal arterial amyloid compared using **a** CRIC, **b** GB, and **c** CRF. The immunohistochemical marker covers and classifies the entire arterial amyloid deposit (**a**), which shows full congruence with CRF (**c**). In polarized light (**b**) only some areas of amyloid are illuminated by GB (see above). Light hemalum counterstaining, ×760

Fig. 3a–c Visualization of amyloid deposits in tissues from the same patient as in Figs. 1 and 2. Glomerular amyloid compared using **a** CRIC, **b** GB and **c** CRF (TRITC filter set). Amyloid is immunohistochemically marked and classified as *AA*, which shows various intensities pointing to variable exposures of the respective antigen determinants. *Arrows* mark weak IC staining in spite of GB and CRF. Light hemalum counterstaining, ×380


1a
1b
1c
2a
2b
2c
3a
3b
3c

rows in Fig. 3a. In addition, CRF is not impeded by the immunohistochemical overlay of the chromogen AEC (Figs. 2c, 3c) or the thickness of the tissue sections (not documented).

Quantitative comparison of CRF with CR and CRIC

In order to compare the sensitivity of CR, IC and CRF quantitatively, CR and subsequently IC staining was applied to the same 396 sections, 128 with (positive control) and 57 without AA amyloidosis (negative control) and 211 sections with variable amounts of AA amyloid, which had been reported to be negative for amyloid during the first historical examination [29, 30].

When these experiments were initiated approximately 6 years ago the results of the readings were variable, in particular with CR but less so with CRIC, while CRF, which had been in routine use for only 3 years, did not reveal significant variability. Whether this difference is due to the sensitivity of the method or reflects a learning effect (or both) has not been examined. In order to eliminate the variability, the readings were repeated until consistency was reached, meaning that the intraobserver variability was virtually eradicated. Thus, the quantitative numbers reported here represent the summary and the end-point of various readings with a minimal intraobserver variability.

The result is shown in Fig. 4. All three methods (CR, CRIC, CRF) showed congruent results only in the control biopsies, whether they contained no amyloid (Fig. 4a) or whether amyloid was present (Fig. 4b). When only small amounts were present, each of the methods resulted in a different proportion of amyloid-positive sections (Fig. 4c).

Statistical analysis revealed a significant difference, as indicated in the legend to Fig. 4.

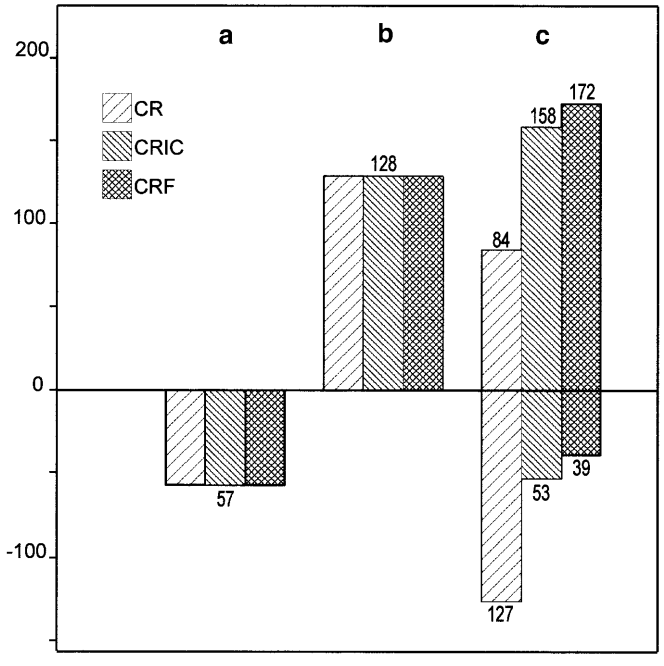


Fig. 4a–c Quantitative comparison of CR, CRIC and CRF in consecutive serial biopsy sections of several patients (see Table 1, patients 2–5). *Ordinate*: Number of sections positive or negative for amyloid. **a** One biopsy (1519,1 of patient 2) negative in all 57 sections for amyloid using the three different techniques indicated by *different cross hatching*. **b** Two biopsies (1519,2 of patient 2 and 1416,2 of patient 3) positive in all 128 sections with all techniques. **c** Four biopsies (1416,1 and 1416,3 of patient 3; 1150,9 of patient 4; 1150,15 of patient 5) variably positive depending on the technique used. The *illustration* indicates increasing sensitivity in order: CR<<CRIC<CRF. Statistical evaluation using the T-test for paired samples revealed significant differences (P<0.001) in all combinations (CR-CRIC, CR-CRF, CRIC-CRF). It is also clear from the amount of amyloid detected in the various biopsies and sections that amyloid is not uniformly distributed within the biopsied organs (shown here on rectum and kidney)

Identification of amyloid classes

As shown in Table 1, the amyloid syndromes of 25 patients examined with CR, CRF, and also in part using CRIC with appropriate antibodies, were immunohistochemically examined and in part confirmed by more direct techniques. Extraction of amyloid fibrils, with isolation of the respective amyloid fibril proteins followed by complete or partial amino acid sequence analysis, was performed in patients 1, 7, 9, 10 (to be published), 12, 14, 19, and 20. Immunohistochemistry using our routine panel of monoclonal and polyclonal antibodies, which is able to identify the common amyloid classes AA, Aλ, Aκ, ATTR, $A\beta_2M$ (for abbreviations see Table 1), resulted in identification of the correct amyloid class in all patients whose amyloid fibril proteins were chemically identified and/or identified by other means. This also applies to patient 13, who had a familial immunohistochemically identified TTR-amyloid syndrome, and in whom DNA sequencing subsequently identified the hereditary amyloidogenic point mutation Val 20 Ile. In patient 14, who was on long-term hemodialysis, the amino acid sequence conformed with the immunohistochemically identified $A\beta_2M$-amyloidosis.

The immunohistochemical results were also consistent with the isotype of a monoclonal light chain in patients 6–10 and 22, and with the clinical syndromes (pediatric systemic inflammations) in patients 2–5. The clinical picture in patients 11 and 12 was also consistent with the systemic senile ATTR amyloidosis identified. In patient 6, two different protein thesauroses, i.e. κ-light chain deposit disease (κ-LCDD, Congo red negative, no GB) and Aκ-amyloidosis (Congo red positive and GB positive), were identified. While both deposits were clearly separated by CRF, in that only the Aκ-amyloid deposits showed bright CRF, they were not separated immunohistochemically, since both kinds of deposits demonstrated immunostaining with anti-Aκ. Amyloid restricted to the skin in patients 15 and 16 with lichen amyloidosis and paresthetic notalgia immunoreacted with anti-keratin antibodies [14], while all antibodies against

the common amyloid fibril proteins were negative. These findings are consistent with the assumption that this type of amyloid is derived from cytokeratin, although definitive sequence data are lacking. Patient 17 had AANF amyloid deposits in the atrium according to the characteristic morphology of the deposits and the sole reaction with antibodies directed against ANF. In patient 18 with Alzheimer's disease, a major reaction was seen with anti-Aβ, and the globular amyloid in a pituitary prolactinoma in patient 21 reacted predominantly with special antiprolactin antibodies (in preparation), while the interstitial amyloid in patient 23 did not react with anti-prolactin or any other antibody used including our routine panel (see above). The amyloid fibril proteins of patients 21 and 23 are therefore chemically different from each other (paper in preparation). Two different amyloids were identified in patient 22, i.e., systemic Aλ-amyloidosis and the organ-limited AIAPP in the islets of Langerhans. Amyloid deposits of patients 23–25 did not react with any of the antibodies included here and most probably represent amyloids still to be identified. It has to be emphasized, however, that CRIC can only be applied when appropriate antibodies are available. Thus, CRIC cannot be used for classification in unknown amyloid deposits.

CRF can be applied to many different amyloid diseases

A total of 28 different tissue samples from the 25 patients having 11 amyloids not cross-reacting immunohistochemically and three unknown amyloid types were examined to answer the question of whether or not CRF can successfully be applied to different amyloids and can assist in the immunohistochemical classification of amyloid. A number of sections were prestained with CR followed by immunohistochemistry for antigenic identification using CRIC (see above). As shown in Table 1, the amyloids examined with CRF included AA, Aλ, Aκ, ATTR sporadic and hereditary, $A\beta_2M$, Aβ, AScr, AIAPP, AANP, ACys, AFib, APro, and three still unknown types of amyloid (for abbreviations see legend to Table 1).

The results reveal higher sensitivity of CRF than of CR. In particular, tiny amyloid spots and amyloid that do not readily pick up CR or show fading with time [32] are detectable with CRF. The increased sensitivity of CRF has facilitated the diagnosis in patients with some immunoglobulin-derived amyloids (patients 7, 8), with amyloids in lichen amyloidosus (patients 15, 16), and with amyloids of unknown origin (patient 23 with pituitary amyloid, patient 24 with "portal amyloid" and patient 25 with "elastic amyloid". In addition, during photography in UV light the CR staining faded considerably. This confirms reports of CR as a light-sensitive cotton stain [32]. As a control, a nonamyloidotic protein thesaurosis (Table 1, patient 6) did not reveal CR staining or CRF illumination while the accompanying Aκ did.

Discussion

The data presented here show that the diagnosis of amyloid deposits in tissue sections is not unequivocal in all circumstances, but may depend on the method of diagnosis used. While sections lacking amyloid are negative with all three methods applied and sections containing large amounts of amyloid are positive in all tests, the same tissue sections with tiny amounts of amyloid are evaluated differently in the same laboratory by the same evaluator, the result depending very heavily on the sensitivity of the method used. In view of the relative absence in the literature of papers on the diagnosis of earliest amyloid deposits, our approach represents a decisive step forward in recognition of minute amyloid deposits, which are usually missed by routine clinico-pathological evaluation. These minute deposits have peculiar properties, some of which have been described here for the first time. This contribution concerns the quality control of the histopathological diagnosis of amyloid and amyloid diseases. This result needs some comment.

While CRIC had previously been shown to be more sensitive than CR [29, 30], as has been confirmed here, CRF has been shown in this report to have the capacity of detecting even a few more small amyloid deposits than CRIC. The sensitivity of the three methods examined can thus be presented as CRF>CRIC>>CR, with the classic CR method as the least sensitive, which can cause false-negative results [29]. The practical consequence will be that every CR-negative tissue section should now be tested with CRF and the congruent GB when the clinician suspects the presence of amyloidosis.

CRF was described and its sensitivity noted in 1953 by Cohen et al. [5], who noticed the detectability of small deposits of amyloid, and confirmed by Cooper [7]. However, both reports described CRF as rather unspecific, and CRF was still reported as "unsuitable for routine use" in 1985 [4]. Nonetheless, the sensitivity and specificity of CRF was described as long ago as 1965 by Puchtler and Sweat [31], but no application for routine use has previously been reported to my knowledge.

The increased sensitivity of CRF is based on the bright yellow-orange illumination that contrasts well with the adjacent dark green background (Figs. 1–3). Another advantage is the ease of application of CRF, since no additional staining is required and the same tissue section and even the same histological detail can be illuminated at will by changing over to either bright, polarized or UV light with the section left in place, as illustrated in Figs. 1–3. To improving the sensitivity of the CR binding further it may be worthwhile to explore other wavelengths of CR or even other amyloidophilic fluorochromes.

The specificity of the CR staining procedure, CRIC and CRF rests *exclusively* on the demonstration of GB after the stringent and selective Puchtler staining [32], which has to be carried out precisely. Thus, these three procedures are not specific for amyloid in themselves but require the demonstration of GB [4, 7, 12, 13, 22, 31].

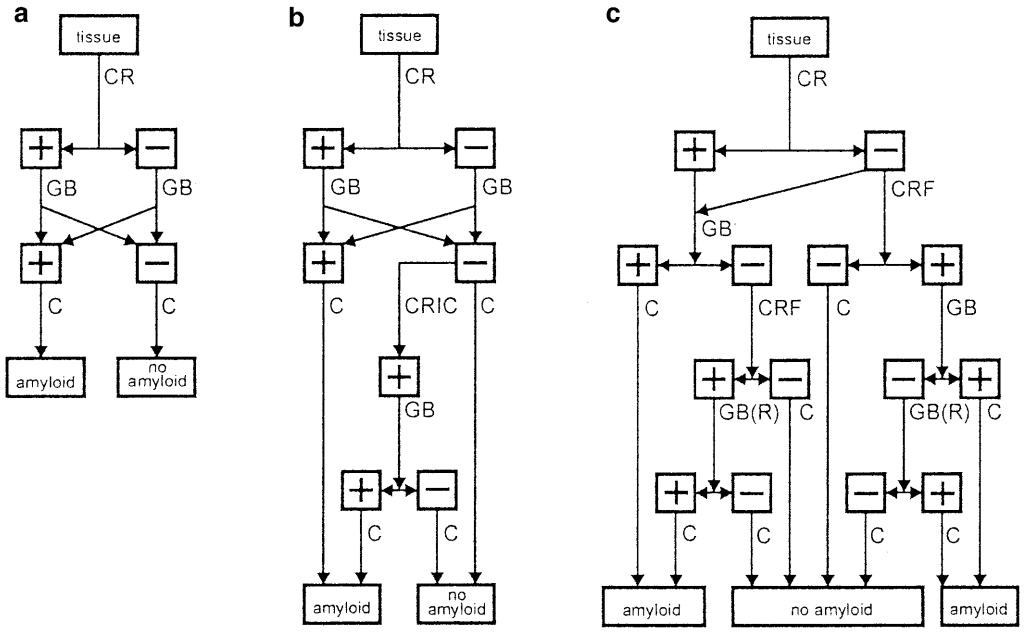


Fig. 5a–c Decision trees to summarize and compare the three methods CR, CRIC and CRF used to diagnose amyloid. **a** Classic CR staining method with examination in bright (*CR*) and polarized light, the latter to search for GB. With the identification of GB the final conclusion (*C*) is drawn as to whether or not amyloid is present. **b** CRIC is applied to examine GB-negative tissue sections. Immunohistochemistry can identify missed amyloid deposits, which are then identified as such via the presence of GB. **c** CRF is applied for examination of small but also weakly CR-binding tissue structures that are regarded as negative for GB by routine examination. Very small CR-binding structures can easily be identified and then scrutinized for amyloid through the recognition of GB if necessary when a rotating slide table (*R*) is used. It should be emphasized here that when no amyloid is detected in an individual tissue section 10 more tissue sections should be examined to exclude a sampling error. In case amyloid is detected CRIC can be applied subsequently for immunohistochemical classification of amyloid in order to identify the protein of origin [29]

As an example, in some sections (Table 1, patients 2 and 15) elastin was found to bind CR intensively and to be illuminated by CRF. In addition, sometimes collagen can pick up some CR that is easily detectable by CRF. Since in both cases the CR-binding areas do not reveal GB, they do not represent amyloid. Another example that could illustrate the usefulness of CRF may be the fibrillar huntingtin deposits in Huntington disease, which are sometimes described as amyloid like. Our own results in three cases (by courtesy of Prof. R. Schröder, Cologne) show that these deposits do not represent amyloid, since they do not stain with CR, do not exhibit GB and do not show CRF. Also, strongly CR-binding detritus from the surroundings of the laboratory or elsewhere can sometimes become attached to the tissue and become stained and embedded. The resulting very strong CRF does not represent amyloid, although in some cases GB can be demonstrated, which may represent cotton threads. In other cases the detritus is seen as intensively white birefringence. Nevertheless, the detritus can easily be separated from the relevant tissue structure by careful inspection, since it is never part of the tissue architecture. A final example relates to the quality control of CR-stained tissue sections, which may be a problem when the staining is not carried out regularly by experienced personnel. Since CRF enhances the visibility of the CR bound to tissues any unspecific binding of CR is easily detectable.

Other fluorochromes, such as thioflavin T and S, are also very sensitive markers for amyloid, but they are described as not specific for amyloid [7, 19, 35]. Only the optical brightener for cellulose Phorwhite BBU has been reported to be specific for amyloid [40], but this reagent has also not been applied routinely.

The reason for missing amyloid in 14 sections using CRIC was reinvestigated in 12 of them: in 3 cases amyloid was detected right away upon examination, while in 5 cases the “polarization shadow” was noticed, which could be overcome by rotating the slide table. This not only emphasizes that a correct diagnosis is guaranteed by such techniques as CRIC and/or CRF, which provide increased sensitivity, but also demonstrates that another factor is just as important: improvement of the examination procedure. The latter should include the use of a slide table that can be rotated for optimal GB illumination of all parts of the tissue section in order not to miss any of the possible amyloidotic protein deposits.

The absence of IC staining in 4 out of 178 sections containing amyloid is remarkable. This unusual and novel finding, which could indicate the inaccessibility of antibodies to tiny amyloid spots, is puzzling and needs further analysis. This finding probably relates to tissue barriers that could be passed by CR but not by antibodies (work in progress).

A summary and a comparison of the three procedures, CR, CRIC and CRF, is presented in Fig. 5. The classic CR-staining procedure (Fig. 5a) can miss small amyloid

deposits when GB is not identified. This may occur when a microscope of poor quality is used, when overstaining with CR or hemalum occurs, and when the sections used are too thick or too thin. Decisive importance also attaches to the specific experience of the individual evaluator [29]. Since minute amyloid deposits may be missed [29, 30], methods with higher sensitivity, such as CRIC (Fig. 5b) and CRF (Fig. 5c) have to be applied. Although CRF can be used to identify amyloid in rare instances where CRIC is negative or no antibodies are available, these two techniques mark possible amyloid deposits before they can be definitely identified by GB at maximal magnification. Since the screening procedure of the whole tissue section at maximal magnification is very time consuming and undermines the observer's full attention, the few relevant areas have to be marked and identified first before they are to be carefully scrutinized for the presence of GB with unwavering attention.

The usefulness of CRF has been shown to be applicable to a large panel of different amyloid classes tested in this report. It has been successfully applied not only to formalin-fixed tissues, but also to the subcutaneous tissue smears in cases of systemic amyloidosis (work in progress), which was pioneered by Westermark [41]. CRF is particularly favorable for detecting amyloids that stain only weakly with CR (Table 1). From this data, it can be deduced that other classes and other varieties of amyloid not examined here will also be easier to detect. CRF should be particularly useful for eradicating sampling error, which has been addressed before [29, 30]. CRF will also be useful in the IC classification of amyloid on CR-prestained sections, since CR-binding areas are easier to detect when unimpeded by the chromogen overlay of CRIC. The easier identification of the congruence of CR (by way of CRF) with GB and the chromogen has considerably improved the specificity of our IC classification procedure of amyloid.

A major result of this study is the characterization of the special properties of minute amyloid deposits, some of which have been described here for the first time. The properties summarized here can help to explain why these deposits are difficult to detect and what can be done to identify them nonetheless.

1. These deposits are usually present in biopsies that have previously been evaluated as amyloid negative in routine clinico-pathological practice because these deposits are very few in number and prone to sampling error [29, 30].
2. These amyloid deposits may be very small and, because of their size, may take up less CR than would be expected of an amyloid slice approximately 4 µm thick.
3. These deposits may be surrounded and shielded by nonamyloidotic tissue, which may weaken the recognition of CR and GB and hamper the access of antibodies.
4. Minute deposits may represent unidirectionally aligned bundles that can easily be missed by a phenomenon for which we propose the term 'polarization shadow.'

The high sensitivity of CRF and the specificity safeguarded by GB also provide a new tool for monitoring patients at risk, in particular gene carriers of hereditary autosomal dominant amyloidoses, the most common being the TTR-derived amyloidoses [38]. Other patients at a high risk who may benefit are patients with juvenile chronic arthritis or other severe and sometimes hereditary inflammatory diseases (familial Mediterranean fever) and patients with a monoclonal gammopathy. In these patients subcutaneous fat aspiration is one of the easiest bioptic options [41]. With respect to monitoring, protein AA has recently been quantified in fat aspirates using a micro-ELISA [3], showing a direct correlation of the amount of SAA-antigenic material with the clinical data. How these quantitative data correspond to the data obtained by radioactive ^{123}I-SAP imaging [15] and CRF in early AA-amyloidosis needs to be examined.

All amyloids should be detected as early as possible and arrested the moment when they are identified, if possible preclinically. A "waiting list" for a life-saving therapy should be as short as possible to avoid further progression of the diseases and (as noted now with a few years of experience) the more or less irreversible organ damage. It seems obvious that the earlier the diagnosis of amyloid can be made and the chemical origin identified, the greater will be the benefit to the patient, in that a rational strategy can be planned and carried out before severe organ damage has ensued [30]. CRF can make a decisive contribution to the achievement of this end.

Acknowledgements For technical assistance, I thank Ms. S. Schade, R. Forster, and R. Joswig. For help with photography, I thank N. Schorner (Fotostelle, MPI für Neurobiologie), and I am grateful to PD Dr. G. Rothe, Regensburg, for the statistical analysis. This paper was supported by the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg, Germany by grants (Li247/7-4, and 12-2) and by the Max-Planck-Institut für Biochemie in Martinsried, Germany.

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