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Pharmacology of Cyclosporine (Sandimmune) III. Immunochemistry and Monitoring

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TWO main reasons prompted the immunochemical study of cyclosporine (CS).* First, CS is a cyclic peptide with a rigid conformation, and hundreds of natural or synthetic derivatives of CS are available. CS represents, therefore, an ideal model antigen for studying the conformational parameters that govern antigen-antibody interactions. Second, the immunochemical study of CS was also required for monitoring needs. Indeed, the use of CS in transplantation and, more recently, in certain autoimmune diseases has been hampered by the large intersubject variability in the CS pharmacokinetics, its narrow therapeutic margin, and its side effects, including the nephrotoxicity that may occur at high concentrations. Therefore, it has been necessary to precisely monitor CS blood levels to adjust individual patient's dosages. The original radioimmunoassays (RIAs) available for CS monitoring were poorly specific for the parent drug because they revealed many of CS metabolites as well. This was due to the high cross-reactivity of the polyclonal antisera used in these assays for some CS metabolites, and the need to improve CS therapeutic monitoring by a more specific assay using monoclonal antibodies to CS was apparent.

* Abbreviations used are: CS, cyclosporine; MAb, monoclonal antibody; MeBmt, N-methyl-(4*R*)-4[(E)2-butenyl]-4-methyl-L-threonine; NMR, nuclear magnetic resonance; RIA, radioimmunoassay; HPLC, high-pressure liquid chromatography.

A. Immunochemical Studies

A precise immunochemical study of the antigenic structure of CS was initiated using highly specific monoclonal antibodies (MAbs) rather than polyclonal antisera which usually exhibit broader specificity.

1. Fine Specificity of MAbs to CS

More than 180 MAbs to CS belonging to IgG, IgM, and IgA classes, and having high affinity for CS (up to 10^{-10}–10^{-11} l/mol) have been prepared (71). The fine specificity of 27 of these MAbs was determined by assessing their ability to react with various CS analogues. A series of more than 60 CS derivatives, substituted singly at each of the 11 amino acid residue positions, was studied (71). Bulky substitutions or derivatives presenting a modification in the peptidic skeleton conformation were avoided. The binding of CS derivatives to MAbs was determined in competitive enzyme-linked immuno-

sorbent assay where the reaction between CS-bovine serum albumin conjugate coated on the solid phase and MAb was inhibited by CS or CS derivatives free in solution.

When the inhibitory capacity of several derivatives modified at the same position was decreased by more than 10- to 100-fold compared to native CS, it was concluded that the modified amino acid residue might be a contact residue involved in the epitope recognized by the MAb on the CS molecule. Conversely, when the capacity of all the derivatives modified at a given position to inhibit the CS MAb reaction was the same as that of native CS, it was assumed that the modified residue was not part of the epitope.

Two main groups of MAbs, issued from different immunization protocols with different immunogenic conjugates of CS-ovalbumin and recognizing different regions of the CS molecule, could be differentiated. Closely related recognition patterns were found in each of the two main MAb groups. Of 18 anti-D-Lys^8-CS conjugate MAbs that were studied, 17 were found to recognize mainly the β-pleated sheet part of the CS molecule (residues 1–6; see section II) which probably corresponds to the most exposed part of the CS molecule on the D-Lys^8-CS-ovalbumin conjugate used for immunization.

Completely different antigenic recognition patterns were observed in the case of MAbs obtained from mice immunized with Thr^2-CS-ovalbumin conjugate. Eight of nine anti-Thr^2-CS-ovalbumin MAbs recognized epitopes involving residues 1, 6, 8, and 9, which are likely to be the most exposed residues on the conjugate used for immunization, whereas residues 2, 10, and 11 were usually not recognized.

In the two series of MAbs studied, the apparent size of the CS antigenic sites recognized by different MAbs varied from 4 to 10 residues and did not seem to correlate with antibody affinity.

2. MAbs as Tools to Probe the Conformation of CS in Aqueous Medium

The influence of CS conformation on MAb recognition was studied using CS derivatives presenting various conformational modifications as determined by ^{13}C nuclear magnetic resonance or X-ray analysis (73). Most of the MAbs were very sensitive to conformational changes, their recognition of CS being gradually decreased as the conformational modifications presented by the molecule increased. For instance, most MAbs recognized CSE, in which demethylation of residue Val-11 induces a contraction of the loop part of CS (92), 10- to 100-fold less well than CS itself. CSH (D-MeVal^{11}-CS), which showed a drastic distortion of the CS peptide ring (97), could hardly be detected even at a molar excess greater than 100- to 1000-fold by most of the MAbs.

In view of these results it was possible to use MAbs for investigating the conformation of chemically defined CS derivatives for which no structural data are available. For instance, most of the MAbs tested recognized the isomeric form of CSH much better than CSH itself (approximately 30-fold discrimination of CS derivative / CS for iso-CSH as compared with >100- to 1000-fold for CSH), suggesting that the conformation of iso-CSH deviates less from that of CS than does CSH. Furthermore, although no X-ray analysis of (2,5,7,8-tetra-N-methyl)-CS was available, extensive conformational modifications could be expected in this derivative where all peptidic bonds are N-methylated, therefore preventing the formation of the hydrogen bonds responsible for CS skeleton conformation. Indeed, this derivative could hardly be detected even at molar excess >100- to 1000-fold by any of the 27 MAbs tested, confirming that its conformation might be drastically modified.

Because CS is a potent immunosuppressor active in biological fluids in vivo, it would be of great interest to know its conformation in an aqueous environment. However, because of its poor solubility in water, the conformation of CS in an aqueous medium could not be studied by nuclear magnetic resonance methods, which require concentrations of the substance of the order of milligrams per milliliter. Until now, the three-dimensional structure of CS has been determined in crystal by X-ray analysis and in solution in aprotic solvents by nuclear magnetic resonance (66, 57; see fig. 1). The two structures obtained differed mainly by the orientation of the 7-carbon side chain of residue 1 (57, 48). Some information concerning the orientation of residue 1 when CS is in solution in aqueous buffer could be obtained using immunochemical methods in which concentrations in the nanogram per milliliter range are detected (73).

For this, a series of 26 CS derivatives modified at different positions in the 7-carbon atom side chain of N-methyl-(4*R*)-4[(E)2-butenyl]-4-methyl-L-threonine (MeBmt) residue 1 were tested with MAbs specific for different regions of the CS molecule. This made it possible to determine the fine specificity of these antibodies for the beginning or the end of the carbon chain of residue 1. All 18 MAbs obtained after immunization with D-Lys^8-CS conjugate, which recognized the β-pleated sheet part of CS from residues 1 to 6, were shown to react only with the first carbon atoms of MeBmt-1 side chain (C3′, C4′, C5′) and not with the last two carbons (C7′, C8′). Conversely, most MAbs obtained after immunization with Thr^2-CS conjugate, which recognized the surface of CS formed by residues 6, 8, and 9, reacted strongly with the terminal carbon atoms (C7′, C8′) of MeBmt-1 side chain.

These results suggested that when CS was dissolved in aqueous buffer, the terminal atoms of residue 1 side chain were not available for binding to antibodies recognizing the surface of the molecule defined by residues 1, 2, 3, 10, 11 but were close to residues 6, 8, and 9 on the opposite side of the molecule. This would indicate that, in aqueous buffer, the side chain of residue 1 is

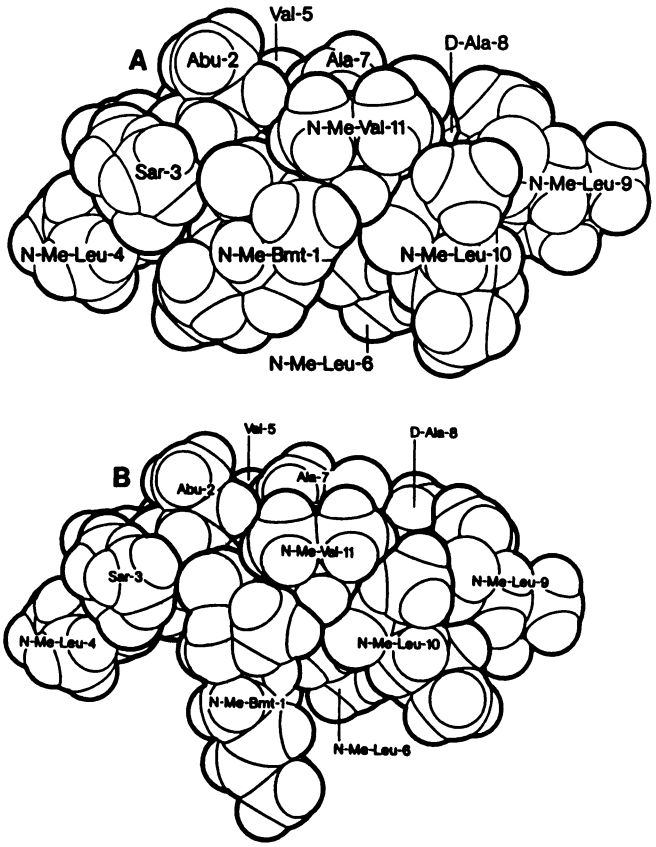

FIG. 1. Identification of the 11 amino acid residues of CS on space-filling models of CS conformation according to X-ray crystallographic analysis (*A*) and to two-dimensional NMR in aprotic solvent (*B*). Abu, alpha-aminobutyric acid; Sar, sarcosine. Adapted from ref. 73 with permission.

probably folded back against the rest of the CS molecule, as observed in crystals, rather than protruding as seen in aprotic solvents. This conclusion is also in good agreement with molecular dynamics calculations which suggest that the crystal structure conformation of CS is more stable than any other in water (reported in ref. 99).

In summary, the immunochemical studies of CS which were initiated by determining the fine specificity and conformational recognition of MAbs to CS will be further pursued with a selected number of these MAbs. The nucleotidic sequencing of their variable regions is in progress (Schmitter et al., submitted manuscript), and X-ray diffraction analysis of crystals of an antibody Fab-CS complex has been initiated (1). These studies, together with the preparation of antiidiotypic monoclonal antibodies (Zenke et al., manuscript in preparation), will certainly make CS one of the best defined peptidic antigens studied so far.

B. Pharmacokinetics

Several reviews of CS pharmacokinetics have been recently reported elsewhere (39, 54, 68, 30, 93, 31, 60, 18, 22, 20, 86). In the following sections, the various aspects of CS pharmacokinetics will be only briefly discussed in relation to CS therapeutic monitoring.

1. Absorption

Large inter- and intrapatient variations in CS absorption have been observed, partly due to the unpredictable bioavailability of CS following oral administration. As a consequence, there was a poor relationship between administrated dose, even when adjusted for body weight, and CS concentration in blood. This has been a strong indication for monitoring CS blood concentrations, especially in the early postoperative phase, to guard against the possibility of insufficient drug absorption.

Several other variables have been reported to influence the oral absorption of CS, including food (still controversial), intestinal disease, diarrhea, liver function, and concurrent therapy with other drugs affecting gastrointestinal motility (see section B.5). CS monitoring has been instrumental in identifying and evaluating the magnitude of such interactions.

2. Distribution

a. In Tissues. CS is widely distributed in many body tissues, the highest concentrations being observed in fat, as expected from CS high lipophilicity in kidney, pancreas, adrenal glands and liver, where it is metabolized (40, 59, 60). A parallel could be established between the wide distribution of CS in body tissues and the presence, in all tissues examined, of the cytosolic binding protein for CS, cyclophilin (51, 83; see section V). However, although cyclophilin seems to be present in brain, very low levels of CS are usually detected in brain, probably due to the inability of CS to cross the blood-brain barrier (60, 96, 68).

Distribution studies revealed that CS has a high tissue affinity (tissue to blood concentration ratios of up to 60-fold in rats; 96) and that it remains in the tissues for several months after discontinuing drug administration (39). Some transfer of CS from blood to milk and saliva, which can be explained by CS lipophilicity, has also been observed (26, 17). In a recent attempt to monitor CS in saliva, only moderate correlation could be observed between saliva and serum CS concentrations ($r = 0.68$, $P < 0.001$; 17).

Because of the high tissue to blood distribution of CS, the relevance of blood concentrations for CS monitoring has been questioned (101). Indeed, no studies have clearly demonstrated yet that changes in the absolute tissue concentration of CS was reflected by parallel changes in blood concentration; however, for practicability, blood (or plasma/serum) remains the preferred biological fluid for CS monitoring so far.

b. In Blood. A concentration-dependent distribution of CS in blood cells has been observed. At CS whole blood concentrations in the range of 100 to 1000 ng/ml, up to 70% of CS could be present in erythrocytes, whereas approximately 20% of the drug is found in the plasma and 5 to 20% is associated to leukocytes (54). The high binding capacity of erythrocytes could be explained by

the presence of a CS-binding protein in human erythrocytes (3), which turned out to be similar to cyclophilin (27; see section V). A consequence of CS uptake by blood cells is that CS concentrations measured in whole blood are always higher than those measured in plasma and serum, usually by a factor 1.5- to 3.0-fold.

CS partitioning between plasma and blood cells was shown to be temperature dependent: when temperature decreases from 37° to 21°C, CS uptake by blood cells increases, and only approximately 50% of the initial concentration remains in plasma (65, 4). CS partitioning seemed also to vary with time between sample collection and plasma separation and with the hematocrit (2, 76, 4, 65).

These variations hampered CS monitoring in serum and plasma. For minimizing the influence of temperature and time of sample separation on CS measurements, most centers using plasma or serum samples adopted standardized separation protocols. Nevertheless, even when using standardized conditions, comparison with other centers using different experimental protocols remained difficult. As a consequence, the members of the Task Force on CS Monitoring, requested in 1985 from the American Association for Clinical Chemistry, recommended that whole blood should be the preferred matrix for CS measurements, primarily for analytical reasons (20).

In the plasma, more than 80% of CS was found to be bound to plasma proteins (54, 65). This binding was independent of CS concentration (between 0.02 and 20 μg/ml in plasma) but varied with temperature (73% at 4°C, 98% at 37°C). The major lipoprotein fractions involved seemed to be high-density lipoprotein and low-density lipoprotein. To assess the clinical relevance of concentrations of free CS versus lipoprotein-bound CS, a study monitoring concentrations of free CS in samples from renal transplants was undertaken (56). No significant difference in the free fractions of CS could be observed before, at, or after nephrotoxicity episodes, leading to the conclusion that measurement of the free fraction of the drug was not superior to measurement of the total blood concentration for CS monitoring (56).

3. *Metabolism*

CS is extensively metabolized by the hepatic monooxygenase cytochrome P-450 system in humans and animals (60). Similar metabolite patterns were observed in kidney tissue and in whole blood in man (78) and in various tissues in rat (59), suggesting that no further CS metabolism occur in peripheral tissue. More than 10 CS metabolites have been isolated from human and animal samples (61). The cyclic peptide ring of CS is preserved in all these metabolites. They present single, double, or triple modifications such as hydroxylation or N-demethylation occurring at a limited number of positions on residues 1, 4, 6, and 9 (fig. 2). Metabolite 17 (hydroxylated on residue 1) and metabolite 1 (hydroxylated on residue 9) are primary metabolites in human and in rat, respectively, together with metabolite 21 (N-demethylated on residue 4). The remaining metabolites seem to be obtained by further oxidation, N-demethylation or intraamino acid 1 cyclization of metabolites 1 and 17.

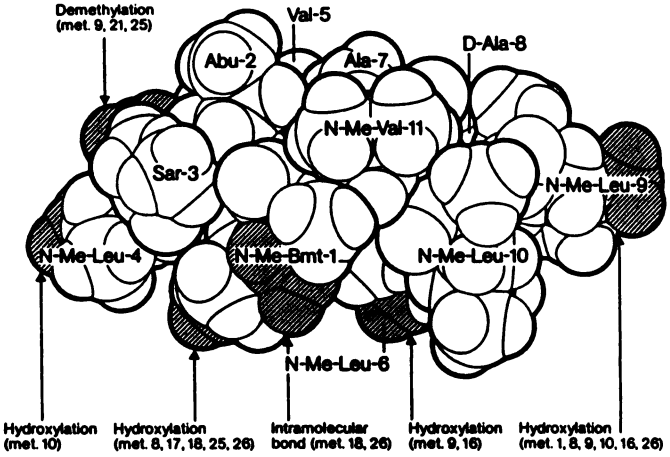

FIG. 2. Three-dimensional model of CS showing the localization of the six main sites of metabolism of CS (according to ref. 60): demethylation and hydroxylation of N-Me-Leu-4 residue, 8′-hydroxylation and intramolecular ether bond of N-Me-Bmt1 residue, and hydroxylation of N-Me-Leu-6 and N-Me-Leu-9 residues. The metabolites modified at the different positions are mentioned. An additional metabolite of CS, in which the 8′-methyl group of residue 1 is replaced by a carboxylic group, has been reported (33). Reproduced from ref. 72 with permission.

In human plasma from healthy volunteers, approximately 50% of CS remains unchanged after single oral administration, whereas metabolite 17 accounts for 15% and the other metabolites (namely, 1, 8, 10, 18, and 21) account for 3 to 7% of the total area under the plasma concentration-time curve (24 hours; 60). However, a different distribution of CS and metabolites was observed in whole blood: CS (27%) > metabolite 17 (24%) > metabolite 1 (14%). All the metabolites tested were strongly taken up by blood cells except metabolites 10 and 18 which were not found in blood cells. The preferential partitioning of CS and metabolites 1, 8, 10, and 17 into blood cells versus plasma has also been demonstrated in vitro (60, 77). Metabolites 1 and 17 seemed to distribute in the cell compartment even more than CS by approximately 20%. In contrast, metabolite 21 was found in a significantly lower proportion in blood cells. The concentration of the different CS metabolites in blood cells could be partly explained by their affinity for cyclophilin, the cytosolic CS binding protein present in red blood cells (27). Indeed, metabolites 1 and 17 were shown to bind to cyclophilin nearly as well or slightly better than native CS, whereas the binding of metabolite 21 was 30-fold lower (70).

The metabolism of CS may be affected by the clinical status or the age of the patient, by concomitant liver dysfunction, or by drug interactions with the enzymes responsible for oxidation of CS. Indeed, much higher concentrations of metabolites have been observed in the

blood of patients with liver, heart, and kidney transplants, where concentrations of metabolite 17 could exceed those of unchanged CS by a factor of approximately 2, varying during the course of treatment (79, 98).

The biological significance of CS metabolites in terms of immunosuppressive activity and nephrotoxicity is still controversial (for review see ref. 83). The potential nephrotoxicity of CS metabolites in rats was evaluated in a parenteral study designed to allow similar bioavailability of CS and metabolites. Because neither renal dysfunction nor morphological lesions could be observed, it was concluded that both metabolite 17 and a whole pool of metabolites extracted from human bile had no nephrotoxic potential in rats. However, because animals display a different metabolite pattern, such results might not be fully transposable to the human situation. More information on the toxic potential of CS metabolites in man could be obtained by careful monitoring of native CS and its metabolites in patients showing different clinical conditions (see below). On the other hand, the immunosuppressive activities of the major CS metabolites were studied in various in vitro and in vivo models (59, 82, 78, 28, 84, 13, 103). In most systems, metabolite 17, which seemed to be the more active of the metabolites tested, exhibited an immunosuppressive activity at least four to 10 times lower than that of CS.

Because CS metabolites can be present at high concentrations in the blood of some patients with transplants and because their toxicity and immunosuppressive activity is not yet clearly established, it has been questioned whether CS monitoring should measure native CS alone or native CS plus its metabolites (this point will be further discussed in section C).

4. Elimination

In man, >90% of intravenous doses of CS is excreted in bile, whereas <6% is excreted in urine. Both in bile and in urine, <1% of the original dose of CS remains as unchanged parent compound (59). It has been suggested that CS metabolites, but probably not native CS, undergo an appreciable enterohepatic recycling process (40, 59). Because of the importance of the liver in the metabolism and elimination of CS, clearance of CS and its metabolites will be reduced by hepatic functional impairment. For instance, in a liver transplant, even when the newly grafted liver is able to convert CS to metabolites, it might be unable to process subsequent elimination, resulting in high accumulation of metabolites (77). Up to 15-fold overestimation of CS level could be observed when the concentrations were measured by immunoassay detecting both CS and some of its metabolites as compared to titration of native CS alone (9). This indicated the need to use an assay specific for native CS with no cross-reactivity for CS metabolites for these patients. However, concomitant measurements of CS plus its metabolites could also provide some important information concerning the role of CS metabolites in various clinical situations.

5. Drug Interactions

CS monitoring has been instrumental in detecting various drug interactions leading to unexpected increased or decreased CS concentrations in patients receiving other drugs together with CS. Several comprehensive reviews of CS drug interactions have been recently published (18, 19, 86). Different CS pharmacokinetic parameters seem to be susceptible to drug interactions. For instance, drugs affecting gastrointestinal motility such as metoclopramide can influence CS absorption, resulting in increased CS bioavailability and CS blood concentration (95). In contrast, the somatostatin analogue Sandostatin, induces a significant decrease of CS blood levels when CS is administrated orally but not after intravenous administration, probably because of its effect on delaying fat absorption (80, 53). On the other hand, phenytoin seems to decrease CS absorption, thereby reducing its bioavailability (81), although it was previously presumed to decrease CS concentration by enhancing its metabolism (44).

Most drug interactions appear to occur via interference with the hepatic cytochrome P-450 monooxygenase system. Indeed, drugs that induce hepatic microsomal enzymes including cytochrome P-450 activity, such as rifampicin (12) or phenobarbital (11), have been shown to accelerate CS elimination, thereby lowering CS concentration. Substances such as the calcium channel antagonists nicardipine (6, 49, 10), verapamil (55), and diltiazem (64, 32, 67, 52) have been shown to increase the CS concentration by interfering with its hepatic metabolism. This might be due to a direct inhibitory effect on cytochrome P-450 in the case of diltiazem (52). Ketoconazole and erythromycin seem to compete with CS for cytochrome P-450 activity, resulting in decreased CS elimination (88) and increased CS concentrations. Indeed, direct in vitro evidence for competitive inhibition by erythromycin of CS metabolism by liver microsomal fractions was reported recently (24).

A higher clearance and a shorter CS half-life were observed during concomitant administration of steroids (68). In patients receiving high-dose steroids, increased CS concentrations could be measured by RIA but not by high-pressure liquid chromatography (HPLC), suggesting an accumulation of CS metabolites. Conversely, CS was shown to potentiate prednisolone therapy by reducing its clearance (69), although normal metabolism of prednisolone in patients receiving CS was also reported (29).

Another type of drug interaction, additive nephrotoxicity, has been reported in the case of drugs having direct nephrotoxic effects or interacting synergistically with CS at the level of the nephron, such as gentamycin, melphalan, or trimethoprim (90, 62, 91).

In summary, CS monitoring has been instrumental in

establishing CS pharmacokinetic parameters and in detecting unexpected drug interactions that affect CS concentration. When CS has to be coadministered with such interacting drugs, CS blood concentrations should be frequently measured together with other parameters such as creatinine levels to monitor the drug interaction. On the other hand, because CS metabolism can be influenced by certain drug interactions, running in parallel a specific dosage of native CS and a dosage of CS plus its bulk of metabolites should be particularly useful to evaluate the actual rate of parent compound over metabolites. Such an assay could be readily performed using MAbs to CS, as will be described in the following section.

C. Monitoring

CS monitoring has proven to be a valuable help in the therapeutic use of CS, primarily because of the unpredictable bioavailability of CS and because CS has a narrow therapeutic margin. In the preceding sections we discussed how some pharmacokinetic parameters of CS, such as absorption, formation and elimination of metabolites, or distribution in tissue and blood compartments, could influence its monitoring. As a consequence, medical centers reported numerous differences in CS monitoring, namely, sample timing, choice of biological fluid, processing of blood samples, and specificity and types of assay used. Several reviews including that of the Task Force on Cyclosporine Monitoring tried to reconcile the immense array of information available (7, 36, 93, 43, 20).

In the following, we will discuss the relevance of specific versus nonspecific measurements for native CS and its metabolites by immunoassays using polyclonal antisera and the newly introduced MAbs to CS.

1. Originally Available Methods

Two types of methods have been available for CS monitoring, namely, HPLCs (reviewed in refs. 7, 68, 20, 93) and immunoassays such as liquid phase RIAs (21, 20).

Although HPLC techniques presented the advantage of detecting only native CS, they usually involved time-consuming procedures and, because of their low sensitivity (usually 25 ng CS/assay), the tests required large blood sample volumes (up to 1 ml). Several highly sophisticated HPLC methods that allow monitoring of individual major CS metabolites have been recently reported (102, 98, 8, 14–16, 87). However, the technical complexity of reliable HPLC measurements restricts CS monitoring by these methods to the few clinical laboratories that possess the appropriate equipment and trained staff.

On the other hand, liquid phase competitive RIAs using tritiated or iodinated CS tracers and polyclonal antisera to CS led to systematic overestimation (2- to 4-fold) of in vivo CS levels compared to HPLC measurements (74). Even higher results (10%) than those obtained in RIAs were observed in a recent automated fluorescence polarization immunoassay using a different polyclonal antiserum to CS (94). This could be explained because the polyclonal antibodies used in these immunoassays revealed both native CS and some of its metabolites, whereas HPLC measures only native CS concentrations.

As mentioned before, CS can be metabolized to variable degrees, depending on the clinical status of the patient. The absence of a strict correlation between RIA and HPLC data was therefore not entirely unexpected and stressed the need for a more specific immunoassay.

2. The Potentials of MAbs for CS Monitoring

a. Rational for the selection of two MAbs for CS monitoring. MAbs can differentiate CS derivatives presenting only minor variations (71), and it was therefore expected that some of them should be able to discriminate between native CS and its metabolites. All CS metabolites that have been characterized from human and animal samples so far presented single, double, or triple modifications occurring only on residues 1, 4, 6, and 9 which are located on the same side of the CS molecule (fig. 2). Because the MAbs are specific for well-defined clusters of amino acid residues on the CS molecule, it was predicted that the MAbs that recognize residues 1, 4, 6, and 9, and are therefore very sensitive to modifications at these positions, should be particularly suitable for discriminating between native CS and most of the metabolites.

Furthermore, because the sites of metabolism are restricted on one-half of the CS molecule, it seemed also possible to select MAbs specific for the region of the CS molecule that is not metabolized (i.e., residues 2, 3, 5, 7, 8, 10, and 11). Such MAbs should be able to detect CS and metabolites equally well and could thus be useful, when used in parallel with MAbs specific for native CS only, for determining the global rate of metabolism in clinical samples.

Two MAbs have been selected out of more than 180 MAbs to CS (72). The first MAb detected 150-fold less well than CS, its main metabolite 17, and >15- to 1000-fold less well all the other metabolites tested (metabolites 1, 8, 16, 18, 21, 25, and 26). The second MAb recognized almost equally well native CS and most of its metabolites. These two MAbs, referred to as the "specific" and "nonspecific" MAbs for native CS, were introduced in liquid phase RIAs using tritiated (^{3}H-RIA; Sandoz Sandimmun-kit) and iodinated tracers (^{125}I-RIA; Inc-Star Cyclo-Trac SP).

b. Assessment of the specificity of the MAb-based RIA in clinical samples. To verify the exclusive specificity of the specific MAb for the parent CS in clinical samples in which CS metabolites are present together with native CS, several studies have been undertaken (e.g., 85, 5). CS dosage was performed either with the specific MAb in ^{3}H-RIA or by HPLC which measures native CS only. Excellent correlations were observed in a large series of

blood samples obtained from normal volunteers (r = 0.97), and patients with bone marrow (r = 0.95), or renal transplants (r = 0.88 and r = 0.94, respectively, in refs. 85 and 5).

More interesting, in blood samples from patients with liver and heart transplants, in whom high metabolite concentrations are usually present, the specific MAb could reproduce the values obtained by HPLC for native CS (r = 0.97). These results, together with those of several similar studies (63, 46), fully confirmed the highly discriminating potential of the specific MAb for native CS versus its metabolites. Immunoassays based on this specific MAb provide convenient measurements of native CS for the clinical centers that do not have access to HPLC techniques.

This specific MAb is being incorporated into several isotopic and nonisotopic semiautomated or automated kits, to unify the dosage data between clinical centers using different techniques for drug monitoring.

c. Performances of RIAs based on polyclonal antibodies and MAbs. To compare the performances of ^{3}H-RIA using the specific and nonspecific MAbs with those of the original polyclonal ^{3}H-RIA, a multicenter study was initiated, involving six centers in Europe and in North America. More than 1300 blood and plasma samples from patients receiving CS following kidney, heart, liver, and bone marrow transplantation were analyzed (35). The median CS concentrations measured by ^{3}H-RIA with polyclonal or specific or nonspecific MAbs for samples of each of these indications are shown in fig. 3.

The results obtained with the original polyclonal antiserum were usually higher than those obtained with the specific MAb but lower than those obtained with the nonspecific MAb. This is in good agreement with the cross-reactivity patterns of the antibodies because the polyclonal antiserum was shown to detect part of the CS metabolites, whereas the specific and nonspecific MAbs could detect virtually none or most, respectively, of the CS metabolites (72). Higher values were generally obtained from liver and heart transplant recipients using the original polyclonal antiserum. This was correlated with the accumulation of metabolites that occurs in these clinical situations, as measured by HPLC of individual metabolites. Even higher values were obtained with the nonspecific MAb, revealing many metabolites that were undetected so far.

When RIAs based on the original polyclonal antisera (or on the nonspecific MAb) were used, it was very difficult to define a unique therapeutic window for renal, bone marrow, heart, and liver transplant recipients. In contrast, similar median concentrations of native CS measured by the specific MAb were observed in the four groups of patients with transplants, indicating that it might be possible to propose a common therapeutic window for all four clinical situations (35). Although the overall correlation between the assay results was highly

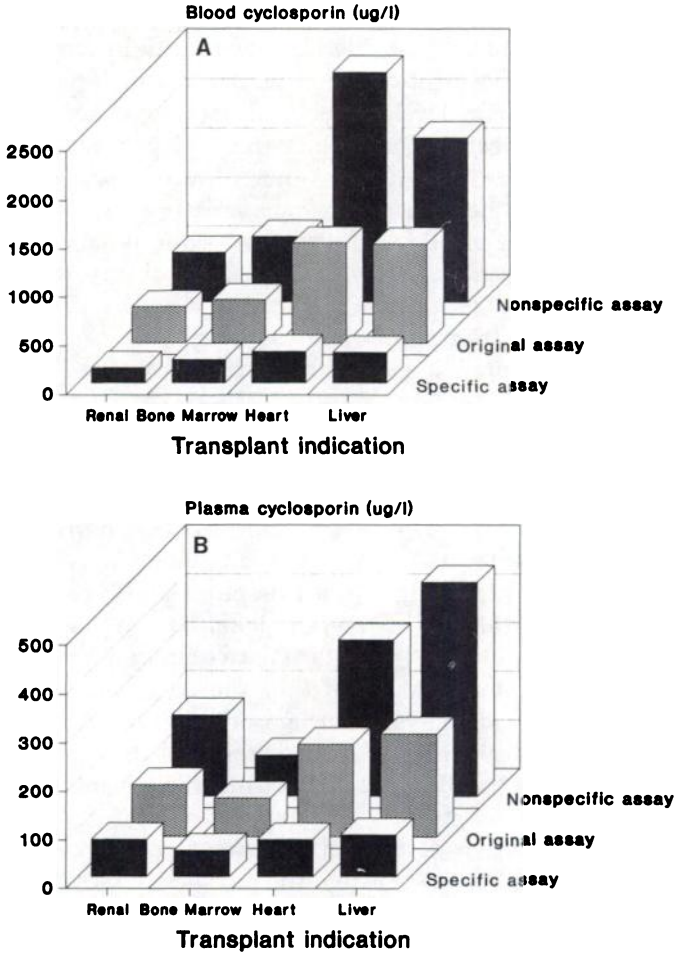

FIG. 3. Median CS concentrations measured by RIA using either the polyclonal antiserum to CS (original assay) or the nonspecific or specific MAbs to CS in four transplant indications. *A*, measurements in blood samples; *B*, measurements in plasma samples. Reproduced from ref. 35 with permission.

significant (whole blood levels 45 to 55% lower being measured in RIA with the specific MAb as compared with the original polyclonal antiserum for renal or bone marrow transplants), the difference between the two assays can vary widely, depending on the biological fluid under study and the clinical status of the patient. This was particularly observed when there were important variations of metabolism such as in liver or heart transplant recipients. It was recommended, therefore, that, for transferring from the original RIA to the specific MAb RIA measurements, individual centers should establish their own within-house correlation and should avoid equating specific with original CS measurements in liver or heart transplant recipients by means of multiplication factors (35).

3. Clinical Relevance of CS Monitoring

The value of CS monitoring depends upon the correlation between measured CS concentrations in blood or plasma and clinical situations. Several authors attempted to relate the events of rejection or nephrotoxicity episodes after kidney transplantation with CS plasma or

blood levels measured by RIA using polyclonal antiserum (table 1). Although considerable overlap did occur between CS concentrations in the two distinct patient groups, mean CS levels associated with rejection were usually significantly lower than those in patients with nephrotoxicity (100, 47, 25). A linear inverse correlation between increment in endogenous creatinine clearance with CS whole blood levels was observed in renal transplant recipients, indicating that nephrotoxicity of CS might be dose dependent (34). On the other hand, after bone marrow transplantation, the rate of development of renal dysfunction seemed to be related to CS serum concentration (42).

Similar results were also observed when CS levels were determined by RIA using the specific MAb in a preliminary study by Taube et al. (manuscript in preparation). However, when retrospective measurements of native CS levels were performed using the specific MAb in patients who had lost their kidney grafts despite high CS concentrations as measured by the polyclonal RIA, it appeared that, in fact, only low amounts of native CS were present. In these instances, knowledge of the data could have helped to avoid the rejection episodes. These findings illustrate the superiority of the specific MAb compared to polyclonal based RIA to detect insufficient immunosuppression.

Some variation of the ratio between measurements obtained with the specific and the nonspecific MAb RIA has been observed. In an attempt to correlate these measurements with clinical situations, Rosano et al. (79) showed that, in patients with either good renal function or rejection, the concentration determined by the nonspecific MAb accounted for the total concentrations of parent compound plus the primary metabolites 17 and 1 (determined by HPLC).

In contrast, a similar study performed with blood samples from a renal allograft recipient with CS-related nephrotoxicity showed that the high concentrations measured by the nonspecific MAb could not be accounted for by CS and the primary metabolites 1 and 17 only (79). Combination of HPLC fractionation and nonspecific MAb RIA were used to reveal as yet unidentified polar metabolites associated with nephrotoxicity. In this example, the ratio between nonspecific and specific MAb RIAs provided an index for the nephrotoxic event (79).

In conclusion, the advent of specific and nonspecific MAbs represents a significant technical and practical improvement in CS therapeutic monitoring. In addition, these two MAbs, together with recently developed HPLC methods that measure individual CS metabolites in blood, provide powerful tools to investigate the role of CS metabolites on clinical immunosuppression and CS associated nephrotoxicity.

TABLE 1

*Plasma and whole blood CS concentrations associated with rejection or nephrotoxicity episodes during the early postoperative renal transplantation period**

Source	Concentrations of CS (μg/liter) associated with:		Refs.
	Rejection	Nephrotoxicity	
Plasma	<150	>300	45
Plasma	<100	>250	41
Plasma		>400	23
Plasma		>500	50
Blood	<200	>800	38
Blood	<200–300		58
Blood	500 (mean)	1000 (mean)	89
Blood	<200–300		75
Blood	<400	>800	37

*CS concentrations were determined in ^{3}H-RIA using polyclonal antiserum.

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