Identifying Clonidine-Displacing Substance

A recent paper by Gen Li *et al.* (*1*) identifies clonidine-displacing substance (CDS) as agmatine. Although agmatine may well displace clonidine from clonidine sites, it differs markedly from the chemical and physiological properties of CDS, which was isolated and characterized in our laboratory in 1984 (*2*). We would like to clear up any potential confusion about the identities of these two compounds.

Clonidine-displacing substance was initially isolated from bovine brain (*2*) and then from serum (*3*) and cerebrospinal fluid (*4*), and has been characterized for its pharmacological properties (*5*–*7*). The isolation procedure of CDS from brain included aqueous and methanolic extractions followed by four successive reversed-phase and sizing high-performance liquid chromatography (HPLC) steps. The final purification step on a C_8 reversed-phase column eluted with water to acetonitrile gradient containing 0.1% trifluoroacetic acid (TFA) (Fig. 1) shows that CDS is retained on the HPLC reversed-phase column, unlike agmatine, which flows through with the void volume.

Characterization of the physical and molecular properties shows that CDS is not a peptide and is heat- and acid (pH 2.0)–resistant, ninhydrin- and fluorescamine-negative, hydrophobic by its retention time on a C_8 reversed-phase column (Fig. 1), and partitions into the aqueous phase in mixtures of chloroform:water and dioxane:water. Agmatine is ninhydrin-positive by virtue of its primary amino group and is not retained on a reversed-phase column.

The estimated molecular mass of CDS determined by size exclusion chromatography was 500 daltons (*2*), and its precise mass was determined by plasma desorption mass spectrometry (PDMS) (*8*). A mass spectrum showed that the molecular ion was m/z 587.8 ± 2, and the appearance of its dimer at m/z 1174 confirmed the monomer's molecular mass (Fig. 2). Agmatine, on the other hand, has a molecular mass of 130 daltons. The molecular mass of CDS was 587.8, which is not a multiple of 130, and "aggregation because of its strong polarity," as suggested by Li *et al.*, is impossible under PDMS conditions. The ultraviolet (UV) spectrum of CDS, which co-eluted with CDS activity, showed the presence of an aromatic chromophore with two peak assignments at 224 and 276 nm (Fig. 3). On the other hand, agmatine is an aliphatic substance with no absorbance in the UV range and thus cannot be CDS, as we initially described. CDS and agmatine have different properties (Table 1).

One unit of CDS activity is defined as the amount needed to displace 50% of 2 nM [^{3}H] clonidine specifically bound to rat brain membranes in a 0.250-ml assay with the use of 10 μM of norepinephrine for nonspecific-binding determination (*2*). An average of 400 units of CDS activity was obtained per brain, corresponding to 4 nanograms per gram of wet tissue (an estimate of the last purification step), as compared with 200 to 400 nanograms per gram of agmatine (*1*). During the purification procedure, the amount of CDS recovered was undetectable spectrally, yet the small amounts were potent in radioactively labeled displacement assays, as well as in pharmacological assays, that is, inhibition of the twitch response in rat vas deferens (*5*), potentiation of human platelet aggregation (*7*), or increasing mean arterial pressure upon cerebral stereotactic injections (*6*). These properties were not shown for agmatine.

In a recent study, CDS and agmatine were tested for reversal of diazoxide inhi-

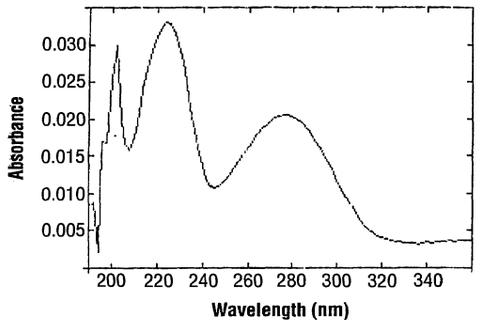

Fig. 3. Ultraviolet spectrum of CDS. The spectrum of the active fraction of the final HPLC step (Fig. 1). Peak assignment was given at 224 and 276 nm. Identical spectra were obtained during all stages of the purification procedure (with the use of a diode array).

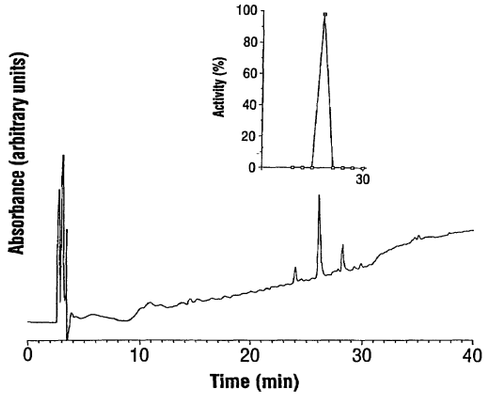

Fig. 1. HPLC profile of CDS. CDS was purified essentially as previously described (*2*). The final purification step was carried out on a C_8 reversed-phase HPLC column and eluted with a CH_3CN/0.1% TFA gradient at a rate of 1 milliliter per minute. Activity of CDS was determined on the lyophilized fractions, as previously described, by a displacement assay of [^{3}H]clonidine specifically bound to rat brain membranes (*2*). CDS activity (shown by the bars depicted above the gradient chromatogram) was eluted at a retention time of 26 min. Absorbance was monitored at 214 nm.

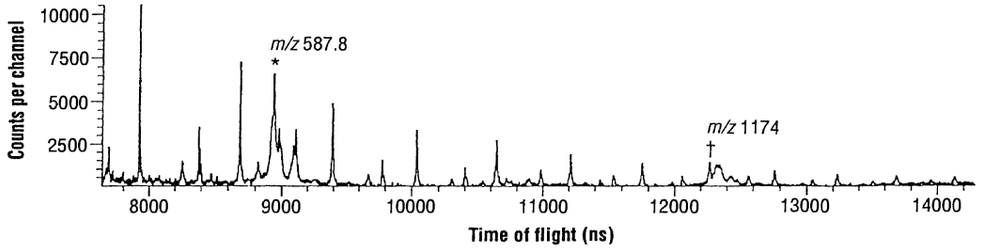

Fig. 2. Plasma desorption mass spectrometry (PDMS) of CDS. CDS eluted from a C_8 reversed-phase column (Fig. 1) was dissolved in methanol, thinly sprayed on aluminized mylar foil, and bombarded with high energy ions (fission fragments) from a californium source (^{252}Cf) (*8*). The molecular weight measured in the field desorption was 587.8 ± 2 (*). The second and smaller peak at m/z 1174 (†) is its corresponding dimer. The sharp peaks are the typical background of the mylar foil.

Table 1. Physical properties and specificity of CDS and agmatine.

	CDS	Agmatine
Abundance/brain	3 to 4 ng/g	200 to 400 ng/g
Absorbance	224, 276 nm (aromatic)	200 nm (aliphatic)
Ninhydrin	Negative	Positive
Molecular mass	587.8 ± 2 daltons	130 daltons
Affinity for α_2AR*	10 to 12 nM	4 μM
Affinity for IR†	20 to 40 nM	1 μM

*α_2AR, α_2-adrenergic receptors. †IR, imidazoline receptors.

bition of insulin secretion in pancreatic islets (*9*): CDS at 2.5 units per milliliter (~12.5 nM) reversed the inhibitory effects of diazoxide, similar to Efaroxan (100 μM), whereas agmatine was ineffective at 10 μm and at 100 μM. Indeed, the affinity of CDS for (i) α_2-adrenergic receptors in rat brain or human platelets was 10 nM (*2*, *3*, *7*); (ii) for imidazoline receptors in rabbit kidney, 20 nM (*10*); in rat liver, 40 nM (*11*); and in human placenta, 23 nM (*12*)—100-fold higher than that shown for agmatine (Table 1).

Although the CDS structure is still elusive at this point, the different molecular and physiological properties of CDS and agmatine suggest that these two endogenous compounds are not the same.

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Response: Clonidine-displacing substance (CDS) was first identified by Atlas as a substance (or substances) found in partially purified bovine brain extracts that competed with ligands for α_2-adrenergic receptors in rat brain membranes (*1*). Confirming her observation (*2*), we also discovered that CDS binds competitively to imidazoline receptors (*3*). To identify the structure of CDS we tracked the ability of purified fractions to inhibit binding of radioligands to α_2-receptors, the same method Atlas used in her purification. We discovered that the substance in brain accounting for virtually all of CDS activity was the amine agmatine (decarboxylated arginine) (*4*).

In her comment, Atlas suggests that agmatine and CDS differ in chemical and biological properties. While this well may be the case, she has yet to establish the structure of CDS, which weakens her arguments with respect to chemical differences and raises questions about whether all or some of the biological actions of partially purified CDS can be attributed to the same molecule responsible for ligand displacement.

Central to Atlas' argument for structural differences is data, summarized in her table 1 and published papers (*1*, *5*), indicating that CDS was first detected by size exclusion chromatography to be approximately 500 daltons and then by PDMS to be m/z 587.8 ± 2. The agent was also said to have an aromatic chromaphore with peaks at 224 and 276 nm, [although in her initial paper (*1*) no UV absorbance was seen at 220 nm]. There is, thus, little new information from this method. Moreover, the comparison between CDS and agmatine was not done side by side.

There are several other problems with this analysis. First, if as Atlas indicates, more than one CDS is present in brain, what is the relative contribution of each? The activity quantitations are based on a measured weight of less than 5 μg. How accurate is this weight? Also, CDS compound is quantified per brain under the assumption that no other CDS-active compounds are present. Second, information is not provided about activity measured at other regions of the HPLC profile. For example, there is no data given about the solvent front where agmatine might be expected to elute. Is there significant activity in this region?

Third, the gel filtration data is convincing, but the mass spectroscopy (MS) data is less so. The HPLC indicates that the material is not pure. Thus, how does one determine that the compound discovered on mass spectroscopy is CDS? What about the range of MS <500 Mr? This lower range is probably complex, but could contain agmatine and related material. Data about this region of the MS spectrum would be desirable.

Fourth, how does one determine that the UV peak is actually CDS? The active material could be co-eluting with a substance that more strongly absorbs UV, but does not have CDS activity. A more general detection method, such as refractive index detection, would be useful to detect a more abundant but non-UV–absorbing material.

Fifth, computation of amounts of units of CDS is based on a ligand-displacement assay. Variations in binding can occur during purification, depending on the conditions of the eluate, which does not easily allow one to compare results between labs or those taken during different phases of the purification cycle for material that can be independently assayed. Hence, comparison of binding constants between CDS and agmatine is not meaningful.

Sixth, it is conceivable that agmatine itself may exist in several forms as a result of its ability to carry charge as bound or aggregated forms modifying apparent size, ninhydrin-positivity, and other molecular properties.

Finally, the possibility of multiple molecules being present in what may appear to be a clean HPLC peak is shown by our data.

The absence of structure also raises questions relating to the biological activities of CDS. Are the actions of partially purified CDS on blood pressure (*2*, *6*), platelet aggregation (*7*), vasoconstriction (*8*), gastric contraction (*9*), or catecholamine release (*10*), as studied by Atlas or ourselves, attributable to a single molecule? If so, is it the same one binding to α_2-adrenergic and imidazoline receptors? Is it agmatine? Complicating the problem is the fact that, as indicated in Atlas' papers, the CDS used for bioassays was not subjected to the same detailed purification steps as used for chemical characterization. Variations in preparations may underlie the fact that microinjection of partially purified CDS into comparable regions of brainstem yield opposite effects on blood pressure when performed by different groups of investigators (*1*, *6*).

Whether the biological actions attributed to partially purified CDS are shared by agmatine is still not clear for the reasons given. For example, the comment by Atlas cites unpublished data that agmatine does not replicate the actions of CDS in releasing insulin from pancreatic islet cells. Others have published that agmatine stimulates (dependent on dose) insulin release and facilitates accumulation of $^{45}Ca^{2+}$ into pancreatic islets (*11*). In our hands agmatine, like CDS (*10*), stimulates (dependent on dose) release of catecholamines from adrenal chromaffin cells. However, unlike CDS, agmatine injected into the aforementioned brainstem sites did not affect blood pressure. Without a defined molecule, biological characterization of CDS is imprecise.

It is possible that a substance or substances other than agmatine may exist in brain or other organs (*12*). This possibility is implied by the title of our report, which indicated that agmatine is "a," not "the," CDS in brain.

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T Cells and Suppression in Vitro

T cell–mediated suppression has been of interest for some time, yet direct demonstration of specific mechanisms has been clouded in part by the diversity of experimental systems. Although suppression is expected to be of most relevance to the prevention of autoimmune disease, for simplicity investigators have relied primarily on the demonstration of T cell tolerance in vitro. Such studies got a considerable boost with the demonstration by Lamb *et al.* (*1*) and Jenkins and Schwartz (*2*) that T cell proliferative responses in vitro might be blocked by inducing T cell receptors to trigger in the absence of co-stimulation. T cells treated in this way became unresponsive, or anergic, to restimulation. Although this mechanism appeared to be of value in explaining peripheral immunological tolerance, issues remained unsettled, including the question of whether inert T cells would be of value to a dynamic peripheral immune system.

Giovanna Lombardi *et al.* (*3*) appear to have resolved this question by demonstrating that T cell clones made anergic by an established protocol could, in mixes with nonanergic T cell clones, block proliferative responses in vitro. The blockade was specific, in that anergic cells were more potent suppressors if they matched the specificity of the nonanergic responder cells, and nonspecific, in the sense that the inert cells could absorb cytokines necessary for driving the in vitro proliferative response.

While we generally support the proposed mechanisms (*3*), we are concerned that these are not necessarily features peculiar to anergic cells in vitro, especially as Lombardi *et al.* irradiated the anergic T cells before adding them to the suppressor cultures (*3*). To test this question, we set up similar studies with the use of T cell receptor transgenic T cells in culture with spleen cells pulsed with peptide that presents antigen. As a source of putative suppressor T cells, we added T cell receptor transgenic T cells that had been stimulated (not anergized) with peptide 48 hours earlier, then irradiated before being added to the culture. These T cell receptor transgenic blast T cells were used because they would be expected to reproduce some of the features demonstrated for anergic T cells: cell enlargement because of blast transformation, retention of cell surface antigen receptors, and increased expression of surface interleukin-2 (IL-2) receptors (*2*); this protocol is similar to that of Lombardi *et al.*, except that the putative suppressor cells are not anergized and are fully functional before irradiation. In the case of T cell receptor–transgenic responder T cells (TCR-HNT) (Fig. 1, A to C), the addition of suppressor cells [irradiated TCR-HNT blasts (Fig. 1B)], at a ratio of 1:1, raised the background counts but still significantly suppressed antigen-specific proliferation. With a second T cell receptor–transgenic responder (TCR-SFE) (Fig. 1, D and E), the inhibition [by irradiated TCR-SFE blasts, (Fig. 1E)] was even stronger. Suppression was specific; so although irradiated TCR-SFE blasts suppressed TCR-SFE responders, they had no effect on TCR-HNT responders (Fig. 1C). Addition of nonlimiting amounts of both HNT and SFE peptide gave dramatically increased proliferation (Fig. 1C, right column).

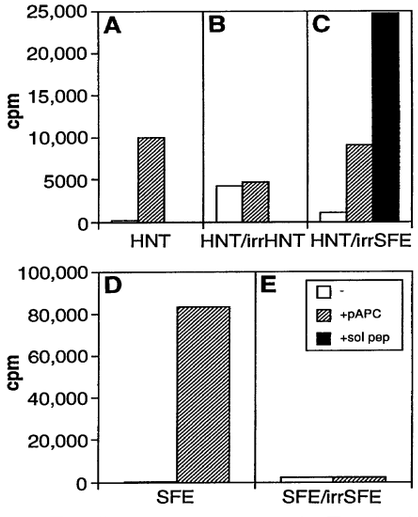

Fig. 1. T cell receptor transgenic T cells from TCR-HNT (*5*) and TCR-SFE (*6*) transgenic mice show specificity for influenza hemagglutinin peptides 126-138 (HNT) on I-A^d or 110-119 (SFE) on I-E^d, respectively. Here, 2×10^5 T cells were stimulated with either 4×10^5 irradiated (2100 R) B10.D2 spleen cells (-), peptide pulsed spleen cells [+pAPC; HNT peptide, (**A–C**); SFE peptide, (**D**, **E**)], or spleen cells in the presence of nonlimiting concentrations of both HNT and SFE peptides (+sol pep, 2 μg/ml each peptide). Bars indicate proliferative responses (cpm, incorporation of ^{3}H-thymidine). T cells (B, C, and E) were cultured 48 hours with peptide and spleen, then washed and irradiated (2100 R) before addition as suppressor cells at a 1:1 ratio to responder cultures (irrHNT and irrSFE).

Our results indicate that, as long as antigen reactive T cells are blocked from proliferation by irradiation, they can act as suppressors of in vitro proliferation. This effect is seen regardless of whether the suppressor T cells are anergic or fully responsive. This point is relevant to the fact that (unirradiated) anergic T cells have been shown to retain the ability to proliferate in response to cytokines such as IL-2, and that the IL-2–driven proliferation will actually induce recovery from the anergic state (*2*, *4*). That is, unirradiated anergic T cells under the conditions described, might contribute to, rather than suppress, proliferative responses. Thus, suppression of T cell proliferation in vitro as described by Lombardi *et al.* is probably not a property specific to anergic T cells, but rather a redemonstration of cold target inhibition.

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Response: The comment by Scott *et al.* is pertinent and thought provoking. They raise the point that the model we propose to account for suppression, mediated by "anergic" T cells, would equally be applicable to the effects of activated T cells paralyzed by irradiation. Data are included in support of his contention.

However, we would like to draw an important distinction between the "anergic" T cells as used in our experiments (*1*) and T cell blasts as used in their system. The stimuli used to induce this anergic state (T