despite their capacity to secrete IL-4 in response to antibody to CD3. These results thus leave open the question of whether early cytokine production by NK1.1$^+$ T cells, by itself, is sufficient to cause Ig class switching or whether conventional CD4$^+$ T cells are important for such help. The earlier work of Goroff *et al.* (*20*), indicating that monoclonal C57BL/6 antibodies to BALB/c IgD can elicit IgG1 and IgE responses in BALB/c mice but not in (BALB/c × C57BL/6) F_1 mice, strongly suggests that conventional T cells capable of recognizing peptides derived from anti-IgD of C57BL/6 origin are generally required for these responses.

The specificity of NK1.1$^+$ T cells for CD1 (*4*) and the demonstration that cells genetically capable of expressing CD1 are essential for restoring the capacity of $\beta_2M^{-/-}$ mice to produce IgE indicate that recognition of CD1 may be essential for activating this pathway of priming for IL-4 production. In mice, CD1 is known to be expressed by cortical thymocytes (*21*) and on gastrointestinal epithelium (*22*). In humans, CD1 is also expressed on epidermal Langerhan's cells (*23*), and a distinct CD1 isoform is expressed on a subpopulation of B cells (*24*). It is possible that the stimuli that elicit IgE production, possibly including infection with helminthic parasites and exposure to various allergens, occur either at sites of constitutive CD1 expression, such as the gastrointestinal tract and the skin, or in response to stimuli that cause increases in peripheral expression of CD1. Such CD1 expression could activate IL-4 production by CD4$^+$NK1.1$^+$ T cells, or possibly by a population of γδ T cells (*25*), at the same time as antigen-specific precursors of T_H cells encounter their complementary ligands. This would provide the IL-4 essential for the priming of such precursor cells to develop into IL-4–producing T_H2 cells and for the development of the type of antibody-dominated immune responses that are characterized by high levels of IgE.

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TECHNICAL COMMENTS

Use of NMR to Detect Water Within Nonpolar Protein Cavities

The structure of human interleukin-1β (hIL-1β) has a nonpolar "hydrophobic" cavity that, in three independent crystal structures (*1*), appears to be empty. On the basis of nuclear magnetic resonance (NMR) data, however, J. A. Ernst *et al.* (*2*) suggest that the cavity contains disordered solvent. They identify protons on the protein that display nuclear Overhauser enhancement (NOE) cross-peaks with water molecules that are purportedly within the cavity. We question, first, whether the water molecules that display NOEs are in fact in the cavity, and second, whether appropriate controls are available showing that NOEs are not shown to protons that are remote from the cavity and from solvent-exchangeable sites.

The protons identified by Ernst *et al.* (*2*) are within side chains near the cavity and include the methyl protons of Leu^{10}, Leu^{18}, Leu^{26}, Leu^{60}, Leu^{69}, Leu^{80}, Ile^{122}, and Val^{132}; the β-methylene protons of Leu^{10} and Leu^{18}; the γ-methine protons of Leu^{10}, Leu^{18}, and Leu^{60}; and the β-methine and γ-methylene protons of Ile^{122}. The NMR experiment does not provide the actual location of the water proton, only that it is relatively close to the protein proton [stated by Ernst *et al.* (*2*) to be less than about 5 Å].

We examined the structure of hIL-1β, as determined by NMR (*3*), to investigate the environments of the protons listed above.

There are, in principle, 26 distinguishable sets of protons. In 18 of these 26 cases we found that at least one proton from each set had a water proton within 5 Å. In four additional cases a water proton was within 6 Å. These water protons are on solvent molecules that form hydrogen bonds to backbone amide and carbonyl groups (*1*) and are of the sort often seen in x-ray crystal structures of proteins. Bearing in mind that the distance calculation included only the seven water molecules identified in the initial NMR analysis (*3*), and that both the x-ray studies (*1*) and the recent NMR analysis (*2*) identify additional bound solvent molecules that were not included, it appears to us that the large majority of the cross-peaks attributed by Ernst *et al.* (*2*) to solvent molecules within the hydrophobic cavity might be a result of water molecules bound elsewhere in the protein.

As a control to the NMR experiment, one would expect that methyl protons that are more than 5 Å from bound solvent, from exchangeable protons, and from the cavity should not display NOEs to water. An example of this sort is provided by Val^{58}, although this is not discussed by Ernst *et al.* (*2*). On the basis of the refined coordinates of Priestle and Grütter (*1*), the two methyl groups of Val^{58} are 5.1 Å and 7.0 Å from the nearest crystallographically observed water molecules. These are carbon-oxygen distances. Because the alignment of the water molecule is unknown, the corresponding proton-proton distances could be somewhat longer or somewhat shorter. (The shortest proton-proton distances to the nearest NMR-identified water are 5.8 Å and 9.0 Å). The methyl groups of Val^{58} are also approximately 10 Å and 8 Å, respectively, from the closest possible waters in the nonpolar cavity and 5.1 Å and 7.2 Å from bulk water at the protein surface. Notwithstanding these apparently long distances, especially for $C^{\delta 2}$, the methyl protons on $C^{\delta 1}$ and $C^{\delta 2}$ of Val^{58} are identified in figure 1A of the report by Ernst *et al.* (*2*) as being involved in direct NOEs with water. The strong NOEs between these protons and water suggest either that distances larger than 5 Å can produce sizeable NOEs or that water molecules have access to other regions of the structure than those suggested by both the crystal and solution structures.

As a further check we calculated the expected oxygen-carbon distances from putative water molecules in the cavity to the side chains that are suggested by Ernst *et al.* (*2*) to make NOEs to cavity waters. The closest approaches that a water molecule of radius 1.4 Å within the cavity of hIL-1β can make to the $C^{\delta 2}$ atoms of Leu^{10}, Leu^{26}, Leu^{60}, and Leu^{80}, respectively, are 5.3 Å, 5.7 Å, 5.3 Å and 6.0 Å, that is, in all cases in excess of 5 Å. These distances are in the same range as seen for the crystallographically observed water molecules described above. Whether the NOEs are made over such long distances, or the NOEs are made to waters that penetrate the entire structure (as compared with Val^{58} above) is an important question with implications both for protein dynamics and energetics. It is to be hoped that further experiments will shed light on these questions. In any event, it remains to be proven that the water molecules that display NOEs with the protons in hIL-1β are necessarily in the hydrophobic cavity.

We do not wish to suggest that nonpolar cavities within proteins are entirely devoid of solvent. The dynamic behavior of proteins allows ready access of nonpolar ligands to internal cavities (*4*) and water molecules to internal sites (*5*). In crystal structures of proteins, nonpolar cavities rarely display electron density that can be interpreted as bound solvent (*6*, *7*). This includes cavities that are large enough to accommodate a water molecule, but sufficiently small to restrict the motion of the water molecule such that it should be readily detectable (*7*, *8*). The widespread crystallographic observation that nonpolar cavities of this size do not contain significant electron density provides strong evidence that the occupancy of these cavities by water is, in fact, low.

Brian W. Matthews
Department of Physics and
Howard Hughes Medical Institute,
Institute of Molecular Biology,
University of Oregon,
Eugene, OR 97403, USA
Andrew G. Morton
Department of Chemistry, and
Howard Hughes Medical Institute,
Institute of Molecular Biology,
University of Oregon
Frederick W. Dahlquist
Department of Chemistry,
Institute of Molecular Biology,
University of Oregon

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Response: We read with interest the technical comment by Matthews *et al.* on our report demonstrating the presence of positionally disordered water within a hydrophobic cavity in human interleukin-1β (*1*). They question whether the NOE and rotating frame Overhauser enhancement (ROE) cross-peaks observed in the two-dimensional (2D) ^{12}C-filtered H_2O-NOE and H_2O-ROE heteronuclear single quantum coherence (HSQC) spectra arise from water molecules that are in fact in the cavity and whether appropriate controls are available.

With the use of the published NMR structure [(*2*), PDB accession number 6I1B], Matthews *et al.* state that in 18 of these 26 cases at least one proton had a water proton within 5 Å, and in 4 cases a water was within 6 Å. With the use of $\langle r^{-6} \rangle^{-1/6}$ averaging, which is appropriate for NMR, we find the following breakdown of distances from these protein protons to water protons: 24 out of the 26 have distances greater than 4 Å of which 8, 9, 5, and 2 are between 4 and 5 Å, 5 and 6 Å, 6 and 7 Å, and greater than 7 Å, respectively. The two protons that are less than 4 Å are Ile^{122}(CβH) and Leu^{18}(Cβ2H). A similar distribution of distances is found upon examination of the three independently solved x-ray structures (*3*). Typically, in ROE spectra one can only see interproton distances up to about 3.5 Å; spin-diffusion peaks, if at all visible, are extremely weak and in the case of secondary effects are of opposite sign to direct ROEs (*4*). Likewise, in a 60-ms NOE spectra of IL-1β, one can only see distances up to at most 4 Å (*2*), and, in addition, the build-up curves did not reveal the presence of any lag phases that would be diagnostic of spin diffusion for any of these 26 groups of protons (*1*). Finally, in the control 2D ^{12}C-filtered H_2O-NOE spectrum recorded with weak presaturation of the water resonance followed by a 200-ms delay prior to the first selective ^1H 90° pulse, all cross-peaks observed in the 2D ^{12}C-filtered H_2O-NOE difference spectrum were suppressed, indicating that they arise from water and not from any proton attached to ^{13}C that resonate in the vicinity of the water resonance (*5*). We therefore conclude that, in at least 16 out of the 26 cases (using a conservative cutoff of 5 Å), the NOEs cannot arise from structurally conserved water molecules participating in bridging backbone hydrogen bonds that have been identified in both the NMR and x-ray structures (*2*, *3*). Given that observed NOE effects, even at an NOE mixing time as short as 60 ms, are of similar intensity to those that involve protons close to structurally conserved waters, we conclude that there is little doubt that the effects we observe originate from water molecules within the hydrophobic cavity. As these water molecules are not visible crystallographically, or more accurately, as the electron density within the cavity is the

same as that of bulk solvent (6), we conclude that these water molecules are positionally disordered.

Matthews *et al.* also state that as a control one would expect methyl protons that are more than 5 Å from either crystallographically identified water, or the cavity should not display NOEs to water. In this regard they cite the methyl protons of Val^{58} for which NOEs to water are observed despite the fact that the γ1 and γ2 methyl groups are 5.1 and 7.0 Å away, respectively (in terms of C-O distances), from the nearest crystallographically observed water molecules, 10 Å and 8 Å, respectively, from the cavity, and 5.1 and 7.2 Å, respectively, from the bulk water at the protein surface. Inspection of the structure, however, reveals that the methyl groups of Val^{58} are only shielded from bulk solvent by the side chains of Met^{44} and Lys^{103}. The distance from the methyl groups of Val^{58} to the $N\zeta H_3$ group of Lys^{103} is too long (>5 Å) to make an indirect mechanism involving an NOE to $Lys^{103}(N\zeta H_3)$ followed by chemical exchange with water likely. Hence, we suggest that the side chains of Met^{44} and Lys^{103} are sufficiently flexible to permit access of water to the methyl groups of Val^{58}. However, not all methyl groups exhibit NOEs to water. For example, no NOEs or ROEs are observed to the methyl groups of Leu^{73} despite the fact that they happen to be on the surface of the protein. A further control is provided by a number of other systems in which these experiments have been used to study water of hydration and in which no NOEs to buried methyl groups were observed unless they happened to be in close proximity to structural water (5, 7).

Finally, Matthews *et al.* state that the closest approach that a water molecule of radius 1.4 Å within the cavity of IL-1β can make to the Cδ2 atoms of Leu^{10}, Leu^{26}, Leu^{60}, and Leu^{80}, respectively, are 5.3, 5.7, 5.3, and 6.0 Å, respectively. However, two additional factors need to be taken into considerations. First, the important distance as far as the NMR experiment is concerned is the distance to the methyl protons and not to the methyl carbon, which in this case will be approximately 1 Å further away from the 1.4 Å radius probe. Second, a water molecule is, strictly speaking, not spherical, and a better probe radius is around 1.2 Å (8); once again it is the distance to the protons and not the oxygen which needs to be considered. Hence, the interproton distance of closest approach is significantly less than 5 Å.

In conclusion, there is little doubt that there is positionally disordered water within the hydrophobic cavity of IL-1β. This cavity, however, is not totally isolated from bulk solvent. As noted in our report (1), there are two small channels (1.9 × 0.4 Å and 1.7 × 1.6 Å in cross-section) that could readily permit penetration of water provided that they expand transiently, and indeed IL-1β displays inherent conformational flexibility (9). In this regard, the cavity in IL-1β is different from those generated artifically in T4 lysozyme which are completely sealed off from solvent (10). While these cavities appear to be empty crystallographically, it remains to be tested experimentally by NMR whether positionally disordered water is present within the T4 lysozyme cavities.

The NMR and crystallographic concepts of occupancy are critical to our paper (1). The widespread failure to observe any significant electron density within non-polar cavities indicates that the occupancy is indeed low in the crystallographic sense. This may be due to one of two factors: (i) either no water is present or water is present for only a small fraction of the time; or (ii) the potential of mean force at any given point within the cavity does not have a well-defined minimum so that water molecules never return to the same position and the electron density is consequently smeared out beyond the level of detection (that is, the water is positionally disordered) (11). If the former were true, no water would have been observed by NMR as the intensities of the NOEs would have been attenuated proportionately. If the latter were true, on the other hand, water would be observed by NMR, as the NMR experiment does not require uniform ordering but is only dependent on spatial proximity, providing the lifetime of the bound water exceeds about 1 ns.

J. A. Ernst
R. T. Clubb
H.-X. Zhou
A. M. Gronenborn
G. M. Clore
Laboratory of Chemical Physics, Building 5, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892–0520, USA

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STM on Wet Insulators: Electrochemistry or Tunneling?

Reinhard Guckenberger and his co-workers (1) describe the use of a scanning tunneling microscope (STM) with high current sensitivity to image DNA on a mica surface in humid air. Because the distance between the STM tip and the sample contact was large in these experiments, current flow and imaging by direct electronic tunneling from the tip to the contact was not possible. We propose that the imaging occurs by an electrochemical mechanism similar to that which occurs in scanning electrochemical microscopy (SECM).

In SECM, the faradaic current produced by an electron-transfer reaction at a small tip can be used to image electronically conductive or insulating surfaces immersed in a liquid phase (2, 3). Usually the sample is placed under a thick liquid layer, and the tip must be sheathed in an insulator (glass or wax). Difficulties in tip fabrication limit their size and hence the attainable lateral resolution. SECM measurements can also be carried out within the thin film of water that forms on the surface of a sample in air, and high resolution can be attained by using tips without insulation (that is, the usual Pt-Ir or W STM-type tips) because the tip area is defined by the small part of the tip that touches the liquid layer. We used a similar strategy to fabricate small metal structures in a Nafion film by SECM, although the attainable resolution at that time was only in the 0.5-μm regime and imaging was not reported (4).

Our instrument can make both SECM and STM measurements with a vertical (z direction) resolution of better than 1 Å and a current sensitivity down to 50 fA with the proper filter (2). The SECM scanning head was contained in a Faraday cage and a Plexiglas box in which the relative humid-