Stereoselective Recognition of Monolayers of Cholesterol, *ent*-Cholesterol, and Epicholesterol by an Antibody

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The interaction between a monoclonal antibody and four distinct monolayers with varying degrees of structural, chemical, and stereochemical similarity were studied and quantified. The antibody, raised and selected against cholesterol monohydrate crystals, interacts with cholesterol monolayers stereospecifically, but not enantiospecifically. Monolayers of ent*-cholesterol molecules, which are chemically identical to cholesterol and whose structure is the exact mirror image of the cholesterol monolayer, interact with the antibody to the same extent as the cholesterol monolayers. The affinity of the antibody for both enantiomeric monolayers is extremely high. However, the antibody does not interact with monolayers of epicholesterol, which is an epimer of cholesterol: The hydroxy group in epicholesterol is in the 3α position rather than in the 3β position, imposing a different angle between the hydroxy group and the rigid steroid backbone, and a different packing of the molecules. Monolayers of triacontanol, a long-chain primary aliphatic alcohol, interact with the antibody to a lesser extent than the cholesterol and* ent*-cholesterol monolayers, presumably due to the structural flexibility of the triacontanol molecule. The lack of chiral discrimination by the antibody is thus correlated to the level at which the chirality is exposed at the surface of the monolayers.*

KEYWORDS:

antibodies · cholesterol · interfaces · monolayers · stereoselectivity

Introduction

Most cellular processes are based on molecular recognition between different molecules, macromolecules, and surfaces. Molecular recognition ranges from chemical recognition (e.g. electrostatic interactions, hydrogen bonding) to steric structural complementarity. Chiral recognition is often a major component of these recognition processes, since natural biopolymers are composed of chiral monomers of unique homochiralities (L-amino acids, D-sugars). Chirality can thus be exploited to distinguish between the various types of interactions determining recognition, because enantiomers have the same chemical and structural characteristics and differ only in their configurations, which are mirror images of each other.

The immune system in particular has evolved such that the binding sites of antibodies are largely variable, enabling remarkable chemical and structural specificity as well as some flexibility. Antibodies are the powerful recognition tool that nature has evolved to tackle potentially any type of foreign invader. The diversity of the immune system is continuously challenged by a wide range of antigens, the response to which is not preencoded. Individual antibodies that have the highest affinity for the target are selected out of the general pool, and their response is subsequently amplified and optimized following the individual challenge. Different antibodies may thus show different levels of cross-reactivity towards stereoisomers and enantiomers.

The question of enantioselectivity of antibodies has been previously addressed. High levels of chiral discrimination were found for polypeptide antigens such as L- and D- rubredoxin,[1] and L- and D- melittin,[2] as well as in the recognition of haptens such as the chiral center of free α-amino acids.[3] Low stereospecificity was observed, however, in other studies of antibody–antigen recognition such as the L- and D-enantiomers of the hexapeptide IRGERA[4, 5] and the stereoisomers of soman (pinacolylmethyl phosphonofluoridate, $C_7H_{16}PO_2F$) a cholinesterase inhibitor.[6] Different antibodies thus show different levels of cross-reactivity towards enantiomers.

It is not surprising that an antibody with a deep binding pocket that envelopes a chiral hapten will show enantiomeric

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discrimination. The issue of chiral recognition becomes, however, far from trivial when the chiral molecules are arranged on a planar surface. The molecular chirality may not be manifested on the surface, such that chiral recognition may not occur. One common example is the surface of proteins in the β-sheet conformation, where the individual chirality of each amino acid is by and large not manifested on the surface of the sheet.[7] On the other hand, the ability of a surface to discriminate between enantiomeric proteins was demonstrated with antifreeze proteins. In vitro studies with synthetic D- and L-enantiomers of an antifreeze polypeptide have shown enantioselective binding to a specific ice crystal face in mirror image directions.[8] In biomineralization, a number of examples of conserved chiral crystal morphologies was interpreted suggesting that chiral interactions between proteins and crystals modulate crystal growth.[9–12] A water-soluble lipase was shown to be inhibited to different extents by monolayers of chiral inhibitors.[13] Particularly relevant to the present work is the chiral discrimination exhibited by the polyene antibiotic amphotericin B, which forms pores in steroid-containing membranes. Amphotericin B produces different ion channels in membranes containing enantiomeric cholesterol than in cholesterol-containing membranes.[14]

The use of crystal surfaces as antigens provides an interesting perspective of the problem of stereochemical surface–surface recognition in biological systems, and in particular in antigen–antibody recognition. Crystal surfaces provide homogeneous antigens whose structures are repetitive and known at the atomic level. The detailed molecular and structural information available on the surface of crystals thus enables a highly sensitive examination of the specificity of recognition and interactions.

Monoclonal antibodies were induced by injection of cholesterol monohydrate crystals and 1,4-dinitrobenzene crystals into mice. Antibodies were selected which specifically interact with each crystal. Two of the selected antibodies exhibited preferential recognition for specific crystal faces.[15, 16] Molecular modeling of the variable regions of these antibodies suggests that the molecular interaction between the binding site of the antibody and the recognized crystal face is based both on geometrical fit and on chemical forces.[17]

The study of the interactions of this antibody with cholesterol crystals was subsequently extended to monolayers of cholesterol at the air–water interface. Epifluorescence studies showed that antibody 36A1 is uniformly and specifically bound to monolayers of cholesterol.[18] The same antibody does not bind under the same conditions to monolayers of epicholesterol.[19]

The interactions between the monolayer antigen and the antibody depends both on the chemical nature of the surface and on structural features. The use of stereoisomers as antigens enables the study of the influence of structural (as opposed to chemical) aspects. The introduction of enantiomeric monolayers allows the same structural arrangement to be studied in mirror image configurations, thus isolating the chiral contribution, if any, to the recognition process. The use of a flexible molecule with the same chemical headgroup provides information on the level of structural adaptability of the antigen–antibody complex. To this end, we have studied and quantified here the interactions of antibody 36A1 with monolayers of cholesterol, enantiomeric cholesterol (*ent*-cholesterol), epicholesterol, and triacontanol, a long-chain primary aliphatic alcohol.

Results

Monolayers of cholesterol, epicholesterol, and *ent*-cholesterol

The three stereoisomers form, at the air–water interface, monolayers with similar characteristics. The surface pressure/area isotherms for *ent*-cholesterol give the same limiting molecular area as for cholesterol, 42 Å^2 per molecule, and the same collapse pressure of 43 mN m^{-1} (data not shown). The limiting molecular area is lower for epicholesterol, 35 Å^2 per molecule, and so is the collapse pressure, 30 mN m^{-1}.[19]

The area per molecule deduced from each pressure/area isotherm was used to calculate the amount of steroid to be spread to achieve coverage of a given interface of the trough. The calculated amounts of each steroid were then deposited in the trough, on the surface of a solution of phosphate-buffered saline (PBS), by evaporation of a chloroform solution of the steroid (5 μL, 10^{-4} M). They were allowed to equilibrate for 15 minutes before observation.

To directly visualize the uncompressed monolayers under the epifluorescence microscope, 1 mol% 3β-cholesteryl-rhodamine-B and 3α-cholesteryl-rhodamine-B were added to the cholesterol and epicholesterol solutions, respectively.[18] The *ent*-cholesterol monolayers were also doped with 3β-cholesteryl-rhodamine-B

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was born in Padova, Italy, in 1950. Her interest in chemistry developed at the Universitá degli Studi di Padova where she received the MSc degree in 1973. She subsequently transferred to the Weizmann Institute of Science in Rehovot, Israel, for doctoral work and received a PhD in structural chemistry in 1979. After a postdoctoral stay at Harvard University, she returned to the Weizmann Institute where she became Associate Professor in 1988 and Full Professor in 1993. Since 1994, she has been Head of the Department of Structural Biology. The unifying theme of her research is molecular recognition of biological components for crystals or other organized molecular assemblies. This includes studies on the mechanisms of biomineralization processes (such as those encountered in mollusk shells, sea urchin spines, bone, and many others), of crystallization processes involved in pathological states, of antibody recognition of crystal and monolayer surfaces, and of cellular adhesion to substrates. In 1998, she was awarded the Prelog Medal in Stereochemistry.

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because the synthesis of the rhodaminated *ent*-cholesterol was technically made impossible by the small amounts available. Under these conditions, all three monolayers retain their fluidity and stability for over 24 hours, and display the same morphological characteristics (Figure 1 A, panels a – c). Homogeneously fluorescent layers are interrupted by small (approximately 10 %) dark regions of empty interface. Doping with 3β-cholesteryl-rhodamine-B does thus not appear to perturb the homogeneity of the monolayer at the resolution available with the epifluorescence microscope.

Observed at the air – water interface, the monolayers are mobile, with rapid random movement of the dark regions. They can be immobilized by lifting onto a glass slide that is lowered from the air parallel to the surface until it touches the interface. Contact angle measurments can be used to verify the presence of a monolayer on the slide, when this cannot be visualized by fluorescence.[19]

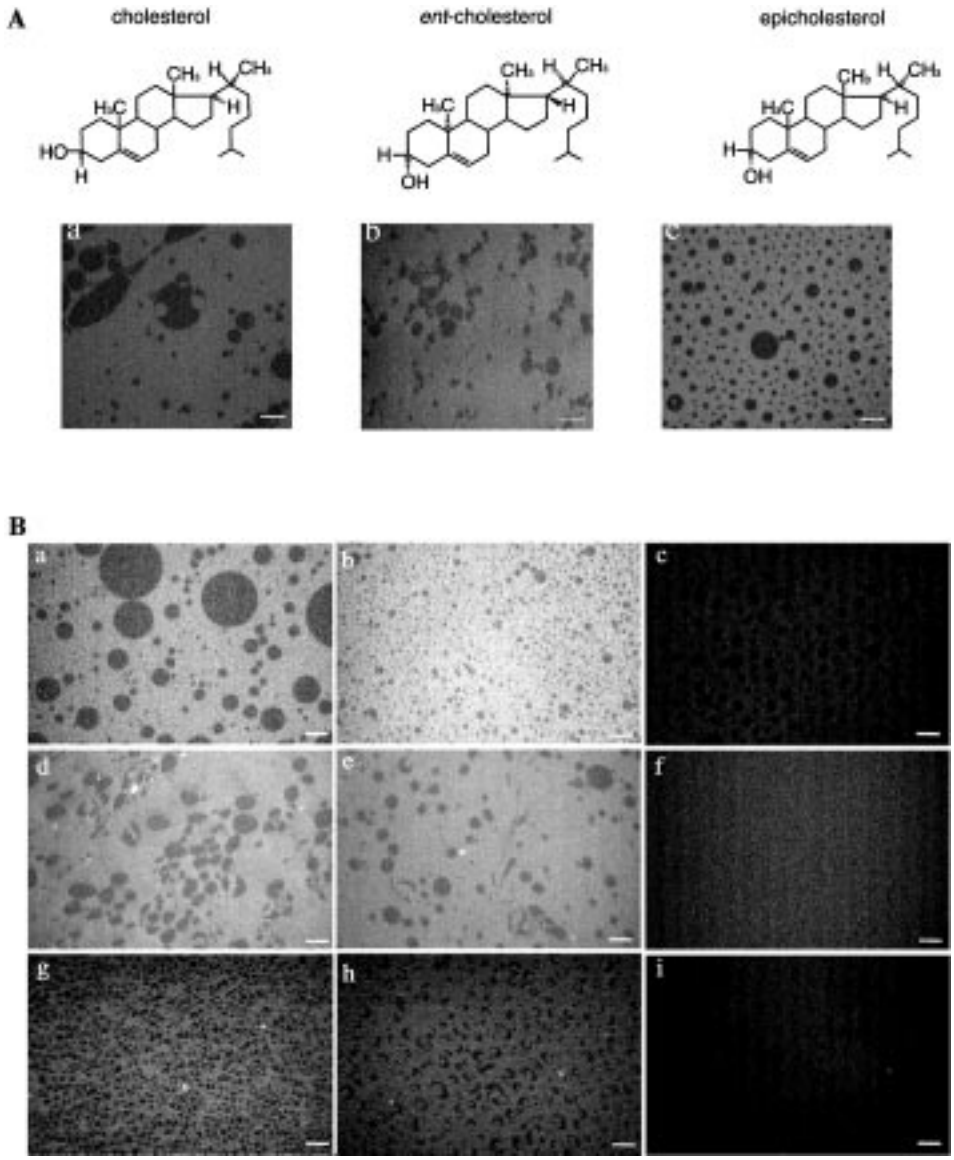

Figure 1. *A: Molecular formulas and epifluorescence micrographs of monolayers of cholesterol (a),* ent-*cholesterol (b), and epicholesterol (c), doped with 1 mol % 3*β*-cholesteryl-rhodamine-B (a, b) or 3*α*-cholesteryl-rhodamine-B (c). Scale bar = 40 μm. B: Epifluorescence micrographs of rhodaminated antibody 36A1 bound to monolayers of cholesterol (a, d, g);* ent-*cholesterol (b, e, h), and epicholesterol (c, f, i). The initial antibody concentrations in the subphase were: 1.0 μg mL^{-1} (a – c); 0.5 μg mL^{-1} (d – f); 0.025 μg mL^{-1} (g – i). Scale bar = 40 μm.*

Interactions of antibody 36A1 with monolayers of cholesterol stereoisomers

To introduce the antibody after monolayer deposition an appropriately devised trough was used, which was equipped with a microchannel system for subphase exchange.[18] Antibody 36A1, an IgM isotype, was purified from ascites fluid by affinity chromatography, fluorescently labeled by covalent binding of rhodamine-B isothiocyanate, and cleaned from excess rhodamine by further purification on a Sephacryl column. The average labeling varied in the various labeling reactions between 5 and 20 rhodamine molecules per antibody molecule. After deposition and equilibration of the monolayers (not fluorescently doped), the fluorescent antibody was injected into the subphase at the desired concentration, and incubated for one hour prior to observation. Any fluorescence observed at the air – water interface under these conditions is due to the interaction of the fluorescent antibody with the nonfluorescent monolayer. A 10:1 excess of a different, nonfluorescently labeled IgM antibody was added as a competitor to reduce nonspecific adsorption of the specific antibody to the monolayers. As an alternative to the use of a competitor, repeated washings of the monolayer with water after incubation with the antibody was used in some experiments. The latter technique was, however, not as reproducible and effective as the former.

To avoid artifacts that may result from antibody aggregation, different environmental conditions, etc., sets of the three monolayers of cholesterol, epicholesterol, and *ent*-cholesterol were consecutively monitored within the same day.

The monolayers were observed first under the epifluorescence microscope directly on the trough and imaged. They were then lifted onto glass slides, and series of images were taken randomly on the slide. Series of eight progressively decreasing concentrations of antibody 36A1, ranging from 1.0 μg mL^{-1} to 0.025 μg mL^{-1} were studied. Representative sets of im-

ages are reported in Figure 1 B at three different concentrations.

At all antibody concentrations, the antibody-bound cholesterol monolayers displayed distinct fluorescent patterns, corresponding to the morphology of the monolayer, while the epicholesterol monolayers were not fluorescent, or showed background fluorescence at higher amplification. At concentrations lower than 0.025 $\mu g\,mL^{-1}$ no distinct fluorescent pattern could be detected on any of the samples. The presence of a monolayer in the epicholesterol slides, when no fluorescence was detected, was verified by contact angle measurements. Antibody 36A1 thus interacts with monolayers of cholesterol but does not interact with monolayers of epicholesterol, ensuring that the fluorescence labeling of the monolayer can be attributed to specific binding.

Monolayers of *ent*-cholesterol, under the above conditions, interact with antibody 36A1 to the same extent as cholesterol monolayers, at all concentrations tested (Figure 1 B, panels a – i).

The amounts of antibody bound per unit area of monolayer at the different concentrations were calculated from the known number of fluorophores bound per antibody molecule, the known area covered by the monolayers, and the observed fluorescence intensity. The antibody concentration in solution, in equilibrium with the complex, was deduced by subtraction of the amount of bound antibody from the amount injected, and is thus an upper limit to the actual residual concentration. The amount of bound antibody per unit area of monolayer is plotted against the residual antibody concentration in solution (Figure 2). Although these data cannot be easily translated into a

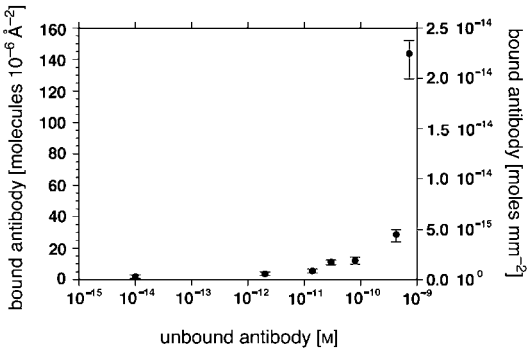

Figure 2. *Binding curve of antibody 36A1, bound to monolayers of cholesterol and* ent*-cholesterol.*

binding constant, because the concentration of effective antigen is difficult to evaluate, they provide valuable information on the very high binding affinity of the antibody for the cholesterol and *ent*-cholesterol monolayers. In particular, these data show that at the lowest concentrations observable, over 90% of the antibody initially introduced in solution is bound to the monolayer.

Furthermore, the interaction of antibody 36A1 with both cholesterol and *ent*-cholesterol monolayers yields a fluorescence image resembling that of the monolayers themselves, in terms of morphology and mobility, implying that the distribution of the antibody is uniform exclusively under monolayer domains. Indeed, the monolayer-free areas at the lowest antibody concentrations (Figure 1 B, panels g, h) appear completely dark even when directly observed on the trough.

We conclude that antibody 36A1 interacts with monolayers of cholesterol and *ent*-cholesterol specifically and to the same extent, not differentiating between the enantiomeric structures.

Interaction of antibody 36A1 with triacontanol monolayers

Next, the interaction of antibody 36A1 with monolayers of triacontanol, $CH_3(CH_2)_{29}OH$, was tested. Triacontanol, a primary alcohol, is chemically different from cholesterol. Structurally, it has a long flexible aliphatic chain, in contrast to the steroids above, which have a very rigid structure. Monolayers of triacontanol were deposited on the surface of a PBS solution as described above.

The interaction of antibody 36A1 with monolayers of triacontanol was studied using the same procedure described for the steroids. A series of antibody concentrations ranging from 1.5 $\mu g\,mL^{-1}$ to 0.2 $\mu g\,mL^{-1}$ were tested. Incubation of the nonlabeled triacontanol monolayer with the fluorescently labeled antibody yielded fluorescence images with distinct patterns of dark and fluorescent areas. These were attributed to a crystalline phase and a fluid monolayer phase, respectively. The crystalline areas appear as rigid shapes with straight boundaries delimited by sharp angles (Figure 3 b). They appear

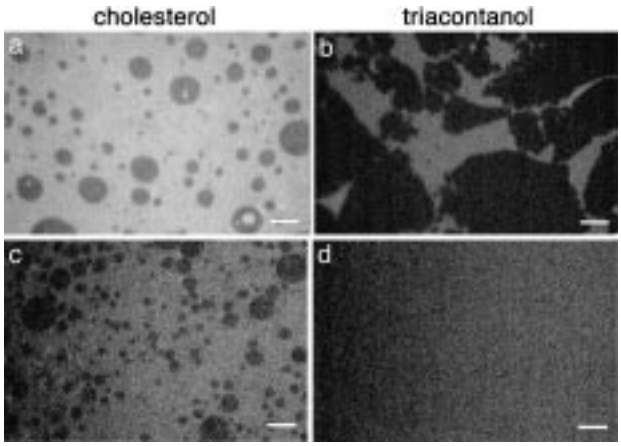

Figure 3. *Epifluorescence micrographs of rhodaminated antibody 36A1 bound to monolayers of cholesterol (a, c) and triacontanol (b, d). The initial antibody concentrations in the subphase were: 1.5 $\mu g\,mL^{-1}$ (a, b); 0.2 $\mu g\,mL^{-1}$ (c, d). Scale bar = 40 μm.*

as dark areas, implying that the antibody does not interact with them. In contrast, the fluid monolayer areas are fluorescent, implying that the antibody does interact with the flexible liquid phase down to concentrations in solution of 0.5 $\mu g\,mL^{-1}$. However, at an antibody concentration of 0.2 $\mu g\,mL^{-1}$, no fluorescent labeling of triacontanol could be observed, while at this same concentration the cholesterol monolayer still preserved labeling (Figure 3 c, d). The presence of the triacontanol monolayer could be detected by higher amplification of the fluorescence signal. The cholesterol monolayer was clearly detected at a shutter speed of 0.16 s, while the triacontanol

monolayer was not. Nevertheless, increasing the shutter speed to 0.32 s a very weak fluorescence pattern could be seen, confirming the presence of the monolayer. We conclude that antibody 36A1 interacts with triacontanol monolayers (not crystalline), although to a lesser extent than with cholesterol monolayers.

Discussion

We have shown here that the recognition between the monoclonal antibody 36A1 and monolayers of cholesterol at the air–water interface is stereoselective, but not enantioselective. Monolayers of both cholesterol and its enantiomer *ent*-cholesterol interact with the antibody to the same extent down to antibody concentrations in solution at least as low as 10^{-11} M. The lack of enantiomeric recognition, although surprising at first sight, may be understood considering the expression of chirality on the surfaces, which is smoothed by the molecular packing. In contrast, the antibody interacts with epicholesterol monolayers only at the highest concentrations tested (1.0 μg mL^{-1}), and even then the interaction is far weaker than with the cholesterol monolayers. When the monolayer is composed of triacontanol, a long-chain aliphatic alcohol, the antibody interacts down to concentrations in solution of 0.5 μg mL^{-1}.

The recognition of the antibody was thus challenged with four distinct monolayers. Cholesterol and *ent*-cholesterol are rigid, chiral, and are chemically and structurally identical, apart from being the exact mirror images of each other. Triacontanol has the same hydroxy functionality, but is chemically different from cholesterol, having an aliphatic chain rather than a steroid backbone, and is thus structurally flexible. Epicholesterol has the same ring system as cholesterol, though it is structurally different: The hydroxy group in epicholesterol is in the 3α position rather than in the 3β position, thus imposing a different angle between the hydroxy group and the rigid steroid backbone. The fact that there is no interaction between the antibody and the epicholesterol monolayer proves that the interaction with the antibody is specific. The fact that the interaction between the antibody and the triacontanol monolayer is weaker than with cholesterol and *ent*-cholesterol, but stronger than with epicholesterol monolayers shows that the interaction is dependent on the structure even more than on the chemical composition of the molecules comprising the monolayer, and is influenced by the rigidity of its molecular components.

Monoclonal antibody 36A1 was raised and selected against cholesterol monohydrate crystals, and was shown to interact preferentially with the {301} faces of the crystals. These faces display molecular steps with one side of the step exposing hydroxy groups and water molecules (as on the {001} faces) and the other exposing the hydrophobic backbone of cholesterol (as on the {101} faces).

The structures of the uncompressed monolayers of cholesterol and *ent*-cholesterol are not known. In compressed monolayers, the molecules were found to form domains with a coherence length of approximately 100 Å, oriented with the hydroxy groups in the water and the hydrophobic backbone forming an angle of approximately 75° to the interface.[20] Therefore, the surface that is exposed to water is expected to resemble the {001} faces of cholesterol monohydrate crystals. At domain boundaries, the hydrophobic backbones may well assume arrangements akin to those exposed at the {*h*0*l*} faces, such that steps with a structure similar to that found on the {301} faces and complementary to the antibody binding site may occur. It is important to emphasize that the monolayer is not static, enabling a high degree of freedom and flexibility relative to crystal surfaces.

The molecular model of the binding site of antibody 36A1 consists of two regions, resembling two faces of the molecular step on the crystal. The hydrophilic part is suggested to interact with the hydroxy moieties of cholesterol, and the hydrophobic part with the steroid hydrophobic backbone. The docking model suggests that one antibody binding site interacts with 10–12 molecules of cholesterol in their structured organization. The hydrophobic face of the 36A1 binding site has five tyrosine residues whose side chains are not completely constrained, enabling at least partial adjustment of their orientation. The differences between the cholesterol and the *ent*-cholesterol {*h*0*l*} surfaces in the crystal are in the different orientations, especially of the methyl groups, relative to the surface. The angle formed in the step and the relative arrangement of the molecules is identical. The {001} faces express very little chirality, if any, whereas the {*h*0*l*} faces do express chirality at the surface. The chiral molecules are structured, however, such that rows of identical groups related by translation are tightly packed together. Inspection of the crystal structure suggests that, because of the tight packing and repetitive structure, the chirality of the individual carbon atoms would not be easily detected upon interaction of an external surface with the molecular arrays.

The results show that the antibody does not distinguish between the structure of cholesterol monolayers and that of its enantiomer. This suggests that the antibody can overcome the minor topographic differences between the hydrophobic enantiomeric surfaces, and interact with the cholesterol and *ent*-cholesterol monolayers to the same extent.

The fact that antibody 36A1 interacts with triacontanol monolayers more weakly than with the cholesterol and *ent*-cholesterol monolayers, supports the above theory. In the case of the enantiomeric surfaces, the flexibility of the antibody's binding site compensates for the minor differences in the structure, whereas in the case of the triacontanol surface, the structure of the monolayer component is flexible, thus enabling interaction. Moreover, the interaction between the antibody and triacontanol is restricted to the liquid monolayer domains, while in the crystalline domains, in which the molecules are constrained in a more rigid structure, there is absolutely no interaction with the antibody. In epicholesterol monolayers, the rigidity of the molecule does not enable interaction with antibody 36A1, because the angle imposed on the step is much more acute.

Interestingly, also the antibiotic filipin, which interacts with single cholesterol molecules, distinguishes between cholesterol and epicholesterol monolayers. The differences in interaction,

however, based on experiments of pressure area changes upon complexion of the monolayers with filipin, show a limited selectivity, much lower than the one observed here.[21]

Cholesterol and *ent*-cholesterol were previously used to study ion channel formation by amphotericin B, proving its enantioselective binding to cholesterol.[14] This result stands in contrast to the lack of enantiospecificity of antibody 36A1, once more highlighting the difference between chirality expressed by single molecules and by molecular arrays.

In conclusion, the antibody recognition depends both on the molecular structure and on the molecular packing. Its stereospecificity appears to be enhanced by the two factors acting together. Judging from the quantitative data obtained here, the affinity of the antibody for both enantiomeric monolayers is extremely high, implying that the chirality of the surface is not a dominating factor in the interaction. The use of enantiomeric and epimeric surfaces provides a tool to achieve keen distinction of the expression of chirality on surfaces, as well as providing accurate information on molecular recognition mechanisms. Once this is established, the high specificity of the antibodies may be conceivably exploited for the design of supramolecular devices such as biosensors.

Experimental Section

Materials and equipment: *ent*-Cholesterol was synthesized as described by Mickus and Rychnovsky.[22] Anti-cardiolipin antibody was provided by Dr. Miriam Blank, Sheba Medical Center, Israel. Cholesterol (> 99% pure), triacontanol, rhodamine-B isothiocyanate, Sephacryl S-200, and dry DMSO were purchased from Sigma (Rehovot, Israel). The ImmunoPure IgM Purification Kit #44897 was purchased from Pierce (Rockford, IL, USA). Cholesteryl-rhodamine and epicholesteryl-rhodamine were synthesized as previously described.[19] Monolayers were imaged under epifluorescent light in a Zeiss (Germany) optical microscope, using a video camera equipped with a MSV-700L integration attachment that allows amplification of the image intensity by a magnification up to $\times 16$ (Applitec, Israel). Absorption measurements were performed in a Ultrospec II spectrophotometer (LKB Biochrom Ltd., Cambridge, England).

Antibody isolation and purification: Antibody 36A1 was raised and selected against cholesterol monohydrate crystals as described previously.[16] The antibody was purified from ascites fluid by affinity chromatography by using an ImmunoPure IgM purification column according to the manufacturer's instructions. The purified antibody was extensively dialyzed against PBS, and stored at 4 °C. Under these conditions the antibody can be stored for up to six weeks without deterioration.

Fluorescent labeling of antibody 36A1: Sodium carbonate buffer (30 μL, 1 N, pH 9) was added to a solution of the purified antibody (270 μL, 0.5 – 0.8 μg mL^{-1}). Rhodamine-B isothiocyanate was dissolved in dry DMSO (40 μL, 1.0 mg mL^{-1}) and was gradually added to the antibody solution over 1 h at room temperature under constant agitation. The solution was stored at 4 °C for 5 h, then NH_4Cl was added (26 μL, 2 N), and the solution was stored at 4 °C for an additional 90 min. The labeled antibody was separated from the unbound rhodamine by gel filtration on a Sephacryl S-200 column. The labeled antibody was stored in the dark at 4 °C. Under these conditions the rhodamine-labeled antibody was stable for up to two weeks, after which aggregation may occur. The rhodamine/antibody labeling ratio was determined for each rhodaminated antibody batch, by measurement of the relative absorption of the antibody ($\lambda = 280$ nm, $\varepsilon = 9.0 \times 10^5$ M^{-1} cm^{-1}) and the rhodamine ($\lambda = 565$ nm, $\varepsilon = 9.2 \times 10^4$ M^{-1} cm^{-1}). The measured ratios were 5, 8, 14, and 20 rhodamine molecules per antibody molecule, respectively.

Immunolabeling of monolayers

Apparatus: All experiments were performed in a $20 \times 20 \times 4$-mm^3 teflon trough, as described by Izhaky and Addadi.[18, 19]

Monolayers: To visualize the monolayers by epifluorescence, 1 mol% of 3β-cholesteryl-rhodamine-B or 3α-epicholesteryl-rhodamine-B were added to the cholesterol and epicholesterol solutions, respectively. The *ent*-cholesterol monolayers were probed with 3β-cholesteryl-rhodamine-B since synthesis of a special probe for the *ent*-cholesterol was technically impossible. Monolayer deposition and lifting were performed as previously described.[18, 19]

Immunolabeling: A monolayer of the required steroid (not fluorescently labeled) was deposited on a PBS solution. After 15 min of equilibration, the subphase was exchanged by injecting the binding solution (3 mL, 1.0 – 0.025 μg mL^{-1} rhodaminated 36A1 in PBS), and the monolayer was incubated for 1 h. The monolayer was then viewed under an epifluorescence microscope to confirm its presence and morphology, transferred to a glass slide, and imaged. At least five images were taken from distinct regions of the slide. Nonspecific adhesion to the monolayer was reduced either by addition of a competitor IgM antibody to the binding solution (10:1 concentration excess), or by extensive washing of the monolayer on the glass slide. Either antibody 48E1 (independently produced in our laboratory against crystals of leucyl-leucyl-tyrosine) or an anti-cardiolipin antibody were used as competitors. Every experiment was repeated at least three times independently.

Quantitative analysis: Solutions of rhodamine in PBS were prepared at concentrations in the range of $10^{-4} - 10^{-8}$ M. For each concentration, a drop of 10 μL was placed between two 24×24-mm glass slides, forming a thin (17 μm) homogeneous layer of fluorescent solution, which was imaged with the epifluorescent microscope. The fluorescence intensity is thus directly correlated to the amount of rhodamine molecules per mm^{-2}. This was used as a calibration for the monolayers' fluorescence by comparing fluorescence intensities. The total amount of antibody bound to the monolayer was calculated by considering the surface area of the monolayer and the number of rhodamine molecules bound to the antibody in each experiment. The residual concentration of unbound antibody in the trough at equilibrium was calculated by subtracting from the total amount of antibody injected into the trough the calculated amount of antibody bound to the monolayer.

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