Monoclonal antibody recognition of cholesterol monohydrate crystal faces

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Background: The immune system can elicit antibodies against a wide variety of antigens. We have proposed that crystal surfaces may also operate as antigens, binding specific antibodies. Here we exploit the crystal surfaces of cholesterol monohydrate to investigate antibody–surface recognition at the molecular level.

Results: Four monoclonal antibodies were selected. Two specifically interact with cholesterol monohydrate crystals, and one with 1,4-dinitrobenzene crystals. The fourth interacts nonselectively with various solid substrates. The relative reactivities of the four antibodies to the different surfaces of cholesterol monohydrate and to other surfaces were compared. The nonspecific antibody adsorbs mainly at imperfections. Of the two specific antibodies, one shows a clear preference for one set of faces, relative to others, the second adsorbs selectively at one face of cholesterol monohydrate crystals.

Conclusions: Monoclonal antibodies can be selected that specifically bind to the crystal surfaces of cholesterol monohydrate. The binding sites of such antibodies appear to recognize a number of molecular moieties, exposed at the surface in a specific structural organization. Different antibodies recognize different structural organizations with varying degrees of selectivity. Antibody–crystal surface interactions may serve as convenient models for studies aimed at an understanding of the molecular bases of antibody recognition.

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Introduction

The recognition of a protein antigen by an antibody involves interactions between the exposed surfaces of the two molecules. Determination of the molecular structure of such antibody–antigen complexes by X-ray crystallography has shown that the topography of the contact area is often quite flat, and that the surface conformation of the antigen does not change substantially upon binding [1,2]. Even though the number of structures of antibody–antigen complexes known is constantly increasing, it is still impossible to systematically analyze and understand the basic rules of molecular recognition underlying the specific interactions between the antibody complementarity-determining region and the protein antigen. A protein surface is highly heterogeneous both in geometry and in chemical nature. Moreover, there are many epitopes available on the exposed surface of the protein, and the selection is not easily predictable. To study the general rules of molecular recognition, it would appear convenient to study a series of antibody–antigen complexes where the antigen is well determined and its variability is under control. Crystal surfaces may answer these requirements. The structures of molecular crystals are easily determined and can be defined at the atomic level. They are characterized by highly ordered, repetitive motifs of molecules. The molecular components of the crystal will be exposed on the different faces of the crystal in different orientations, exhibiting different chemical moieties in a known pattern.

It appears that both the organization of the antigen and the spatial orientation of its epitopes are indeed important for antibody recognition. For example, an anti-virus antibody showed a stronger response when the glycoprotein antigen on the outer membrane was presented in a highly organized repetitive form [3]. Similarly, an anti-phospholipid antibody was shown to interact with its antigen only when the latter was organized in the hexagonal phase, and not in the lamellar phase [4].

We have proposed that, in principle, the flat and rigid surfaces of molecular crystals can act as antigens, eliciting the production of specific antibodies [5,6]. Antibodies against nonprotein antigens containing repetitive patterns have been characterized in other systems such as polysaccharides [7], synthetic polymers with repeating arrays of identical units [8] and viruses [3]. These repetitive antigens generally elicit a 'T-cell independent' immune response, in which the response to the antigen by the antibody-producing B cell is direct, and does not require presentation of the antigen to T cells via MHC molecules [9]. Such a mechanism may be active in the production of antibodies against crystals.

In the case of a crystal surface antigen, each epitope would be constituted by an array of molecules over an area identified as the antibody-binding site. In protein–antibody complexes the area that an antibody covers on a protein is typically ~600–900 Å^2 [1,10]. This is the area covered by an array of 7–25 molecules with a cross section of 5 x 5–10 x 10 Å each. If this array can bind antibodies with high affinity, then the antibody must be recognizing a repetitive series of molecular moieties in the correct arrangement. On each crystal face, the recognition sites would thus have well defined chemical characteristics, which differ one from the other, and from those of the component molecule. We note that such specific interactions between functionalized domains on protein surfaces and a number of matching moieties on specific crystal faces have been observed with proteins other than immunoglobulins in different systems where crystals are exposed to biological environments [11–13].

We have shown that the immune system can respond to the introduction of a foreign crystal in the organism by producing specific antibodies [5,14]. Polyclonal IgG raised against three different crystals (monosodium urate monohydrate, magnesium urate octahydrate and allopurinol) each catalyzed the nucleation of the respective crystals *in vitro*. Similarly, IgG isolated from the joint fluid of patients suffering from gout, a disease associated with the presence of monosodium urate monohydrate crystals in joints, selectively catalyzed the nucleation of monosodium urate monohydrate [5]. This was explained by assuming that some antibodies developed against the crystal faces bear in their binding site a structured imprint of a number of molecular moieties exposed on the crystal surface. These antibodies may thus act as stabilizing templates in a new crystallization event. Although the component molecules of the three crystals are quite similar, little or no cross-nucleating effect was observed, indicating a high level of complementarity between the antibodies and the crystal surface structures [14].

To further elucidate the antibody–crystal interactions at the molecular level and evaluate the level of specificity that can be reached in antibody–crystal interactions, monoclonal antibodies are required. Here we chose to raise and examine monoclonal antibodies against cholesterol monohydrate crystals. This choice was motivated by the fact that cholesterol, in the living organism, may aggregate or precipitate in pathological situations as crystals (gall stones, atherosclerosis). As the molecule is not charged, we predicted that nonspecific adsorption would be reduced, increasing the chance of obtaining specific antibodies. The crystal furthermore presents an interesting variety of surface structures, on which specificity can be tested at high molecular resolution. Finally, generation of monoclonal antibodies against a different cholesterol-containing system, cholesterol-rich liposomes, has been described by Swartz *et al.* [15], providing a convenient closely related system for comparison.

Three monoclonal antibodies that strongly interact with the surface of cholesterol crystals were selected and characterized by various immunolabelling techniques. They present an interesting range of recognition levels, from nonspecific to highly specific. One of them was shown to interact selectively with a single crystal face of unique character.

Results

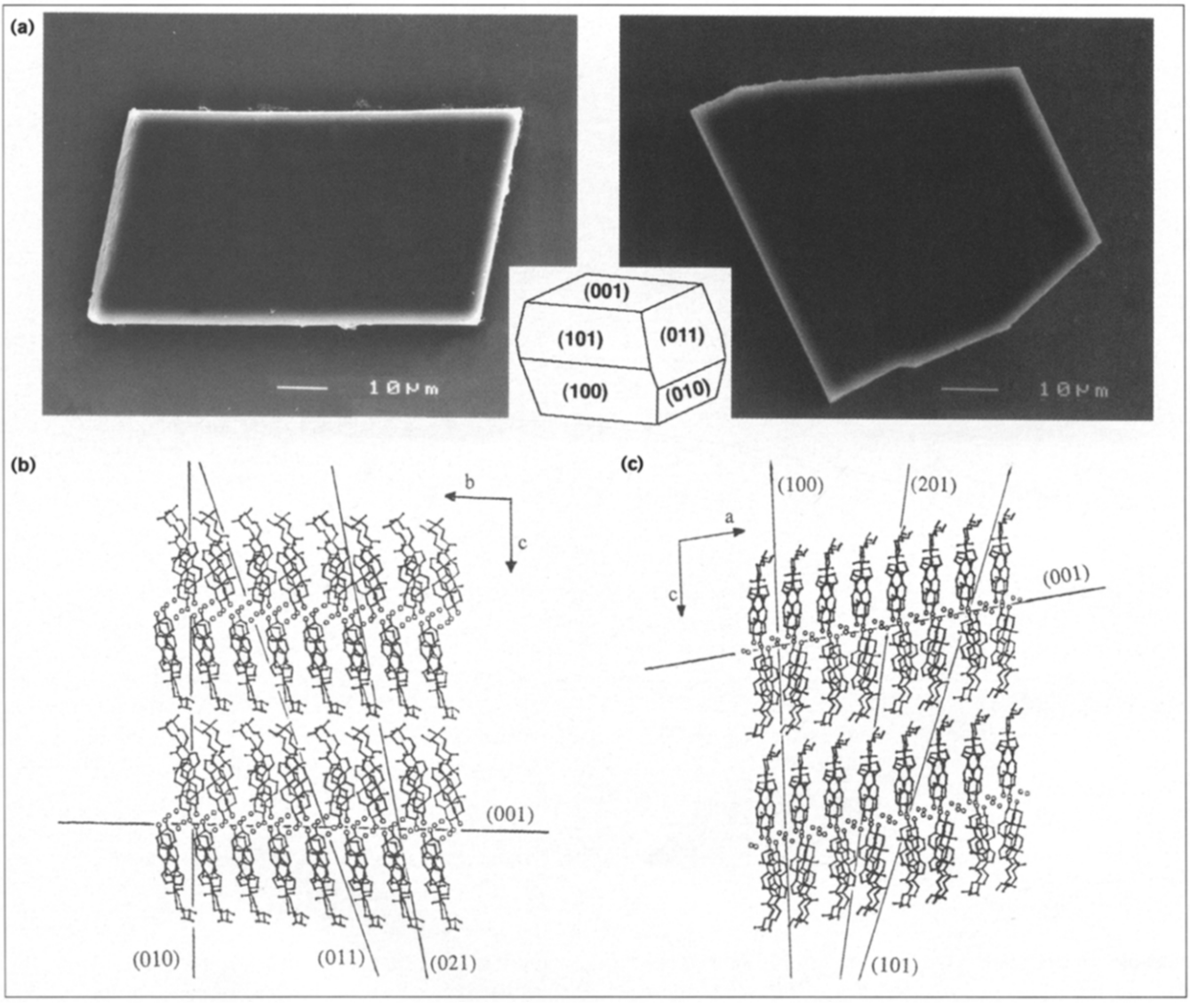
Cholesterol monohydrate crystals and antibody selection

Cholesterol monohydrate crystals were obtained from supersaturated cholesterol solutions of 20 % water in acetone. These crystals exhibit smooth {001} plate faces, as in the typical morphology [16], but faces from the {*h*0*l*} and {0*kl*} families are also well developed. (Note: The notation {h,k,l} indicates the family of symmetry-related faces or planes, (h,k,l) indicates one member of the family, and [h,k,l] indicates the direction of the vector perpendicular to the plane.) The number and indices of these faces vary among crystals. The basic crystal morphology is presented in Fig. 1a. Cholesterol monohydrate crystallizes as bilayers, exposing along the {001} plane the hydrophobic tail of the molecule or the hydroxyls and lattice water molecules in alternate layers (Fig. 1b,c). The {001} faces may thus have hydrophilic or hydrophobic character, depending on the environment to which they are exposed. In water, the hydroxyl–water layer is preferentially exposed, and the face consequently has a hydrophilic character. The {*h*0*l*} and {0*kl*} faces expose the cholesterol molecular backbone and have a more hydrophobic character. All the {*h*0*l*} faces with $l \neq 0$, and {0*kl*} faces with $l, k \neq 1$ have molecular steps where the water molecules and the cholesterol hydroxyl groups are exposed. In contrast, the {100} and {011} faces, which do not contain such steps, are completely hydrophobic.

Two crystal types were used for immunization, cholesterol monohydrate and 1,4-dinitrobenzene (DNB). The latter molecule, when linked to a protein carrier, is a well-known immunogen. Crystals of DNB expose at their surfaces benzene and nitro groups in various arrangements, and are very different from cholesterol crystals.

Following immunization with the two crystal types, monoclonal antibodies (MAbs) were selected on the basis of their relative affinity to the two crystals. The characterization of these hybridoma clone populations is reported elsewhere [17]. Four clones were analyzed here in depth. MAb 36A1 and 58B1 interact strongly with cholesterol crystals, but not with DNB crystals. MAb 122B1 interacts with 1,4-DNB crystals, but not with cholesterol crystals, and was used as a negative control. MAb 23C1, an antibody obtained from a mouse exposed to 1,3-DNB crystals, adsorbs strongly to both types of

Figure 1



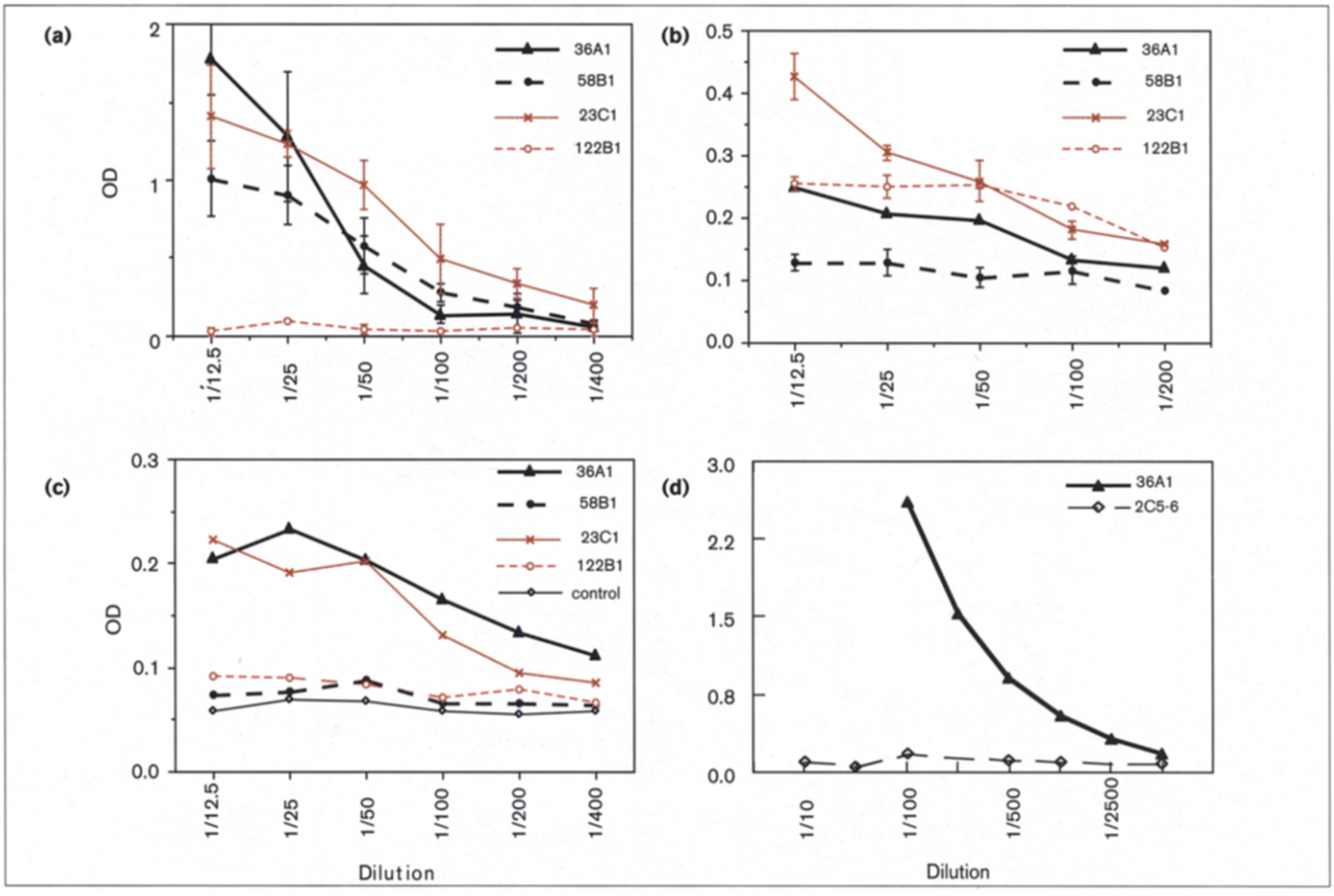
The structure and morphology of cholesterol crystals. **(a)** Scanning electron micrographs of cholesterol monohydrate crystals grown from acetone/water, 80/20. Inset: typical morphology of cholesterol monohydrate crystals grown under these conditions. **(b)** and **(c)**: Crystal structure of cholesterol monohydrate; (b) viewed in the [100] direction or (c) viewed in the [010] direction [24]. The H atomic positions are not marked. C = ●, O = ○. The planes that are developed as stable faces are indicated.

crystal surfaces, and is considered nonselective. A commercial monoclonal IgM antibody selected against cholesterol-rich liposomes (2C5-6) [14] was used as an additional control. MAb 36A1 was obtained after immunization with cholesterol monohydrate crystals, MAb 58B1 and 122B1 after immunization with 1,4-DNB crystals. All the MAbs selected were of IgM type. Interestingly, one of the cholesterol-binding antibodies originated from the population obtained after immunization with DNB crystals [17].

Binding of the antibodies to solid substrates

The selected monoclonal antibodies were first characterized by enzyme-linked immunosorbent assay (ELISA), examining their binding to three types of solid surfaces: crystals of cholesterol monohydrate, crystals of 1,4-DNB, and the polystyrene surface of microtiter plates. The crystals were incubated with sequential dilutions of antibody in the presence of a blocking agent, 0.5 % bovine serum albumin (BSA). In the absence of blocking agent, higher binding was observed, presumably due to non-specific adsorption of the antibodies to the crystal surfaces. In a representative experiment with MAb 36A1, the optical density (OD) in the presence of BSA was 0.4, and in the absence of BSA was 0.7. The results of the ELISA are shown in Figure 2. The monoclonal antibody selected as cross-reactive, 23C1, indeed bound strongly to all three solid surfaces examined. In contrast, MAb 58B1 and 122B1

Figure 2



Monoclonal antibody binding to solid substrates. Binding of monoclonal antibodies 36A1, 58B1, 23C1 and 122B1 to: **(a)**, **(d)** cholesterol monohydrate crystals; **(b)** 1,4-DNB crystals; **(c)** a polystyrene microtiter plate. The reactivity is measured in a solid-state ELISA ($\lambda = 402$ nm).

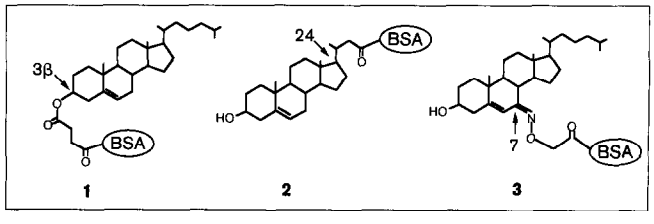
Each value represents the average of at least two reactions at the indicated dilution of hybridoma medium in (a), (b) and (c), and of ascites in (d). Note the differences in OD scale among the graphs.

bound with high selectivity to cholesterol monohydrate and 1,4-DNB crystals, respectively. MAb 36A1 bound weakly to the hydrophobic polystyrene surface but showed overall selectivity for cholesterol monohydrate. MAb 2C5-6 did not bind to cholesterol monohydrate crystals under our experimental conditions. The selected hybridomas thus appear to be sensitive to the chemical and structural composition of the crystal surfaces. The concentrations of IgM in the different hybridoma media were similar (data not shown), except for MAb 2C5-6 where the medium contained a lower concentration of IgM. Ascitic fluid of 2C5-6 was therefore also examined (Fig. 2d).

Antibody binding to noncrystalline cholesterol

We next addressed the question of whether the monoclonal antibodies recognize the molecular components of the crystal, or a repetitive pattern of the component molecules on crystal faces. The first indication of this is intrinsic to the experimental set up of the crystals. All experiments with crystals must be performed in cholesterol-saturated solutions to avoid crystal dissolution, as cholesterol has a low but significant solubility in 0.5 % BSA-enriched buffer. Binding to the crystal is thus always studied in competition with binding to molecular or BSA-associated cholesterol present in solution. To further test the reactivity of the antibodies to molecular cholesterol, BSA–cholesterol conjugates were prepared. Cholesterol molecules were attached to BSA through the hydroxyl group in position 3β, through the

Figure 3

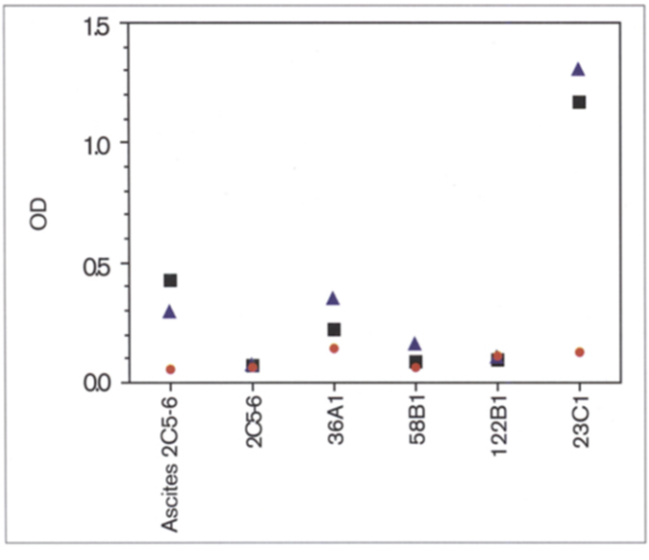


BSA-cholesterol conjugates. **1** Cholesteryl hemisuccinate; **2** 5-cholenic acid-3β-ol; **3** 5-cholesten-3β-ol-7-(O-carboxymethyl)oxime.

hydrophobic tail in position 24, or in position 7 (Fig. 3), using two different synthetic procedures. On the average 8 or 13 molecules of moiety **1**, 5 or 4 molecules of moiety **2** (in the two procedures) and 4 molecules of moiety **3** were attached per BSA molecule respectively. Binding of the selected antibodies, including MAb 2C5-6, to the polysterene-adsorbed BSA conjugates, was then tested by ELISA. The antibodies bound to the cholesterol–BSA conjugates with the same affinity as to the carrier protein treated in the same way but without conjugation. There was thus no binding to the cholesterol moieties.

Taking a different approach, cholesterol was adsorbed onto polystyrene microtiter plates, by drying ethanol solutions containing 0, 1 and 10 μg of cholesterol per well respectively. The cholesterol molecules are presumably present on the plate in different aggregation states. Only a few microscopic crystals were observed with 1 μg of cholesterol per well, while significantly more crystals were present at 10 μg cholesterol per well. Binding of monoclonal antibodies to cholesterol attached to the plates was then examined (Fig. 4). MAb 58B1 did not recognize cholesterol deposited on the plate, whereas MAb 36A1 bound slightly to preadsorbed cholesterol. The cross-reactive MAb 23C1 reacts strongly with cholesterol under the same conditions. MAb 2C5-6, raised against cholesterol-rich liposomes, bound to cholesterol adsorbed on polystyrene plates, as reported [15]. However, the response was higher with 1 μg cholesterol deposited in the well than with 10 μg. These results, in conjunction with those obtained for adsorption onto the crystals, indicate that MAb 2C5-6 binds to some type of adsorbed cholesterol aggregates, but not to cholesterol crystals.

Figure 4



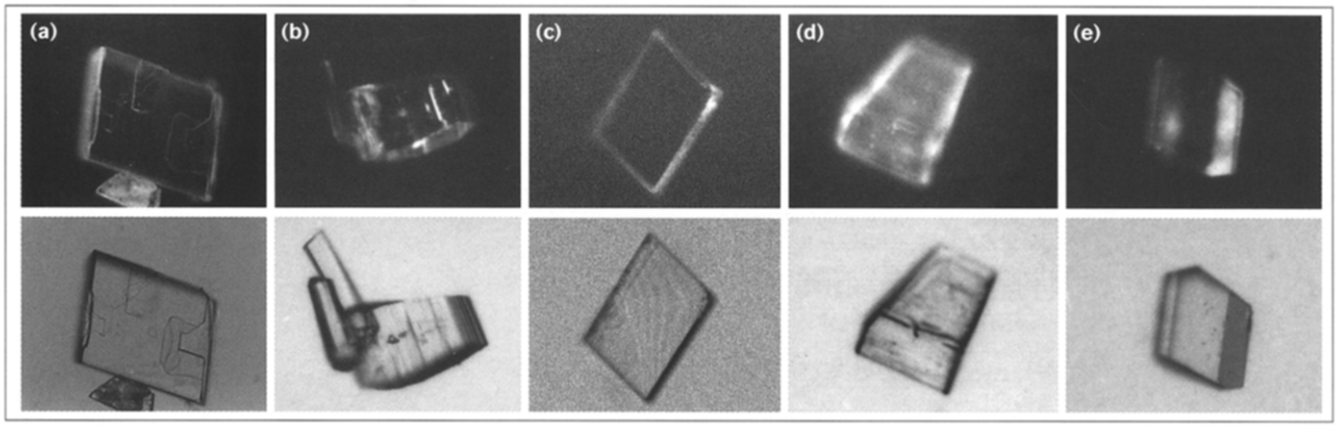
Binding of monoclonal antibodies to cholesterol adsorbed on tissue culture dishes. The amount of cholesterol per well was varied. ● denotes 0 μg, ■ 1 μg and ▲ 10 μg of cholesterol per well. Binding was measured by ELISA, λ = 402 nm. Each value represents the average of two reactions with undiluted hybridoma medium, or with ascites diluted 1/10. Ascites was used for 2C5-6 as the concentration of IgM in the corresponding hybridoma medium is relatively lower than in other hybridoma media.

Immunolabelling experiments

Each different face of cholesterol monohydrate crystals exposes a surface with a distinct chemical and structural character. An antibody that recognizes a defined molecular pattern is thus expected to interact differently with the various crystal faces. Determination of the distribution of antibodies on the different crystal faces would allow definition of the molecular patterns recognized. Experimentally, mapping of antibody on crystal surfaces was performed using immunofluorescence and immunogold labelling. Goat anti-mouse secondary antibodies labelled with rhodamine were used in immunofluorescence studies. The cholesterol crystals appeared under the light microscope either as parallelograms (when the {001} faces are viewed), or as polygons (when their {*h*0*l*} or {0*kl*} faces are viewed) (See Fig. 1a). We note that the {*h*0*l*} and {0*kl*} faces have a layered structure and contain imperfections. Crystal imperfections are high energy binding sites and constitute favored locations for nonspecific interactions. BSA was thus used in the binding assays as a blocking agent. In control experiments, crystals incubated with dansyl-labelled BSA exhibited fluorescence from all faces, especially from crystal imperfections, indicating that BSA indeed behaves as a nonspecific blocking agent (data not shown). Crystals incubated with MAb 23C1 in the presence of BSA exhibit a strong fluorescence, originating mainly from the imperfections on all crystal faces (Fig. 5a, b). In contrast, following incubation with MAb 36A1 and 58B1, the fluorescence uniformly emanated from the sides of the crystals that are lying on their (001) face (Fig. 5c). This is not due to an optical artefact, as observation of the crystals lying on their side faces confirmed that homogeneous fluorescence indeed emanates from these faces (Fig. 5d). When monoclonal antibodies were omitted, the crystals were hardly fluorescent. This unambiguously demonstrates preferential binding of monoclonal antibodies 36A1 and 58B1 to the {*h*0*l*} or {0*kl*} faces. Many crystals were examined. In some of the crystals incubated with MAb 36A1, one of the faces exhibited a much stronger fluorescence than the other faces (Fig. 5e).

Under the light microscope, it is neither possible to distinguish whether crystals are viewed along the a^* or b^* axes, nor to assign the indexes of the faces viewed. At this stage we therefore could not ascertain whether the antibodies recognize particular faces of the {*h*0*l*} and {0*kl*} families. This problem was solved by examining crystals labelled with 30 nm colloidal gold beads (coated with secondary antibodies) with a scanning electron

Figure 5



Immunofluorescence labelling of cholesterol crystals with antibodies. Bright field (bottom panel) and fluorescence (upper panel) micrographs of cholesterol monohydrate crystals, incubated with monoclonal antibodies and labelled with goat-anti-mouse $F(ab)_2$ rhodamine-conjugated secondary antibodies. Magnification is 160x. **(a)** View of the (001) face of a crystal incubated with MAb 23C1. **(b)** View of the (*h*0*l*) or (0*kl*) faces of a crystal incubated with MAb 23C1. **(c)** View of the (001) face of a crystal incubated with MAb 58B1. Crystals incubated with 36A1 appear the same. **(d)** View of the (*h*0*l*) or (0*kl*) side faces, following incubation with MAb 58B1. **(e)** View of the (*h*0*l*) or (0*kl*) side faces of crystals incubated with MAb 36A1. Note that the exposure times were not equal, and the fluorescence intensities are thus not comparable.

microscope. In the scanning electron microscope, the crystal faces were exactly identified by measuring the dihedral angles they form with the easily identifiable (001) plate face (see Materials and methods). The concentration of gold beads on each face was then determined by counting the beads in a set of frames (three or four for each face) at a magnification of 20 000x. Three to six crystals were examined for each orientation ({001}, {*h*0*l*} and {0*kl*}), for each antibody. Each value reported in Figure 6 thus represents the average count of at least nine frames. The frames were deliberately chosen in regions of the crystal surface presenting very few imperfections. It is still possible that the antibody may be associated with imperfections, such as kinks or steps, of a size lower than the resolution of the instrument. At a magnification of 20 000x, the resolution of visible steps is ~10–20 nm. If the antibodies were concentrated along parallel steps, however, one would expect to observe rows of gold beads in the direction of the steps, even if the size of the step was smaller than the resolution of the instrument. No such alignments were seen.

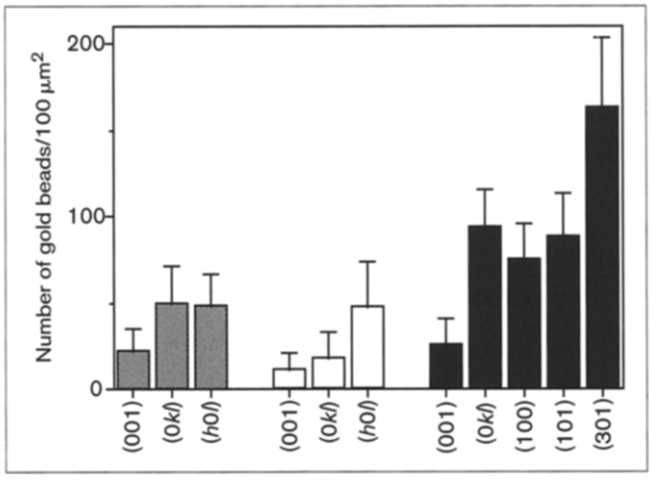
For all three monoclonal antibodies studied, the extent of gold labelling is lower on the {001} faces. The relative difference in label intensity was less pronounced with antibody 23C1. This antibody appears also to give a lower response, relative to the other antibodies, than would be expected from the ELISA results. The counting of gold beads does not take into account gold beads concentrated at macroscopic imperfections, however, where immunofluorescence showed that this antibody tends to concentrate. Macroscopic imperfections are not monitored, because frames containing them are not flat, making counting impossible.

The overall response of monoclonal antibody 36A1 was much stronger than that of 58B1, as already observed in the ELISA experiments. Comparison of the binding to lateral faces for antibody 58B1 shows that the average number of gold beads bound to the {*h*0*l*} faces is 2–3 times higher than on the {0*kl*} faces. No clear difference was, however, observed between the faces on the same side of the crystal. In contrast, observation of the {*h*0*l*} faces of the crystals incubated with 36A1 showed one particular face, (301) or (401), populated with an extremely high number of gold beads. This high order face is very close to the (100) face. The latter, (100), forms an angle of 81° with the (001) face, the former two, (301) and (401), of 76° and 75°, respectively. The presence of {401} or {301} faces could only be ascertained thanks to crystals where both (100) and (401)/(301) faces were present. Since (401) and (301) faces are geometrically very close and have similar chemical characteristics, we cannot distinguish between them and will refer to them as {301} faces. Interestingly, the (100) and (301) faces behave very differently with respect to adsorption of antibody 36A1 (Figs 6,7). Approximately twice as many gold beads are attached to the (301) face than to any other of the lateral crystal faces. {301} faces are not always present, and the result is thus in agreement with the immunofluorescence studies where one face was occasionally, but not always, seen to fluoresce more strongly than the others.

Discussion

In this study, we have shown that monoclonal antibodies can be selected that specifically recognize the surface of cholesterol monohydrate crystals. These antibodies do not seem to recognize the cholesterol molecule, but

Figure 6



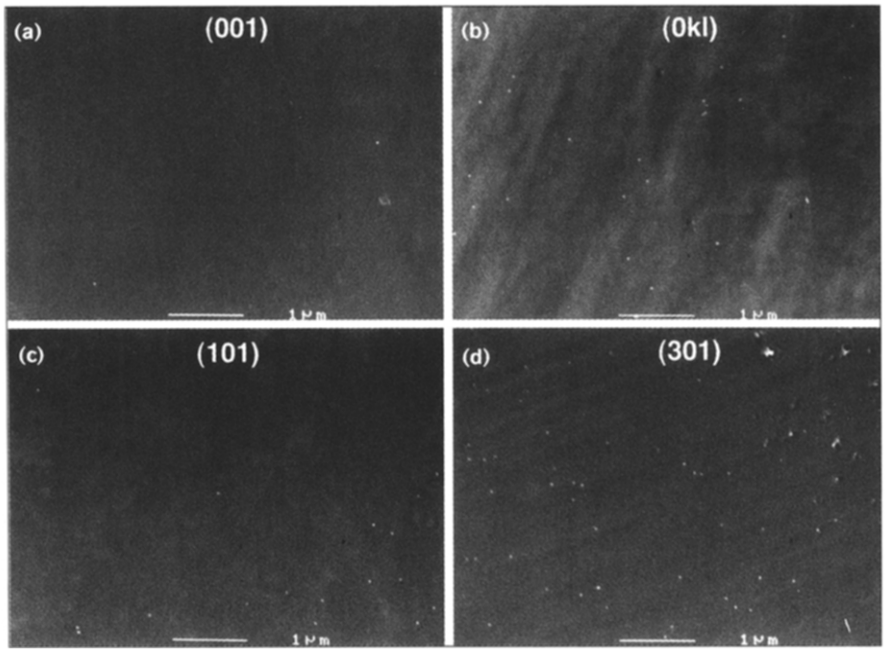
Comparison of immunogold-labelling counts of MAb 23C1, 58B1 and 36A1, adsorbed on the different faces of cholesterol monohydrate crystals. Gray, 23C1; white, 58B1; black, 36A1. Each value represents the average of counts performed in at least nine frames.

rather defined molecular motifs exposed on some of the crystal faces.

Cholesterol is a fundamental component of cell membranes, and the question of whether it can be immunogenic has attracted much interest over the years. The cholesterol molecule has generally been shown to be nonimmunogenic or poorly immunogenic [18]. In contrast, cholesterol-rich liposomes (containing more than 70 % cholesterol) where the cholesterol molecules are presumably not present as isolated entities but in organized domains, can elicit the formation of specific antibodies [15]. The monoclonal antibodies selected against such liposomes were shown to recognize cholesterol adsorbed on polystyrene plates as well. One of these antibodies, MAb 2C5-6, was tested in our system. Under the conditions of our experiment, MAb 2C5-6 did not recognize cholesterol monohydrate crystals. Its reactivity also decreased when a higher amount of cholesterol was deposited on the plate, although the higher amount resulted in a significant increase in the number of microscopic crystals formed. We therefore believe that MAb 2C5-6 recognizes a form of aggregated cholesterol that is present in the liposomes and in plate-adsorbed cholesterol, but not in the strictly defined and organized surfaces of cholesterol monohydrate crystals. In contrast, the monoclonal antibodies selected in this study recognize crystalline cholesterol, specifically a given pattern of epitopes present on one or more of the crystal faces. Such binding is always measured in competition with binding to molecular or BSA-associated cholesterol present in solution. Furthermore, none of the antibodies bound to cholesterol–BSA conjugates. It could be argued that the cholesterol adducts are not exposed to the antibodies when BSA is adsorbed to the plate; as the molecule was bound to the carrier protein through three different positions, using two synthetic procedures and in different reaction environments, this seems unlikely, however. We believe that the absence of reactivity to noncrystalline forms of cholesterol reflects a real feature of the antibodies. It also agrees with the reported failure to directly raise antibodies against molecular cholesterol [18].

Figure 7

Scanning electron micrographs taken from different faces of cholesterol monohydrate crystals, immunogold labelled with MAb 36A1. **(a)** (001) face, **(b)** (0*kl*) face, **(c)** (101) face and **(d)** (301) face.



Upadhya *et al.* [19] reported that IgM and IgA isolated from the bile of patients that form gallstones accelerate the formation of cholesterol crystals. Assuming that nucleation is based on antibody–crystal complementarity, as proposed by Kam *et al.* [5], this would suggest the presence in these bile secretions of antibodies that recognize the surface of cholesterol crystals. The possibility of obtaining specific antibodies that recognize groups emerging at the crystal surface, but not the component molecule of the crystal, was also demonstrated recently for 1,4-DNB crystals [17]. Interestingly, MAbs reactive with 1,4-DNB crystals were produced only after immunization of mice with 1,4-DNB crystals, and not after immunization with cholesterol crystals.

All the MAbs selected for binding to cholesterol and 1,4-DNB crystals were of the IgM type. This is not surprising because crystals are most likely to generate a 'T-cell independent' immune response, resulting mainly in the production of IgM. In addition, the higher valency of the IgM molecule will significantly enhance its binding to the almost infinite array of antigens present on the crystal surface, by virtue of a cooperative effect.

Selection of antibodies that specifically recognize cholesterol monohydrate crystals was carried out on the basis of their ability to bind to the surface of the cholesterol monohydrate crystals, but not to other unrelated surfaces. Such a procedure is essential since macromolecules tend to adsorb nonspecifically onto surfaces. Upon adsorption to solid surfaces, proteins are also likely to denature to some extent [20,21]. The adsorption may thus be partially or totally irreversible. The presence of a blocking agent, BSA, at a high concentration, should reduce these nonspecific interactions. Even in the presence of competitor, we observed that some monoclonal antibodies do bind very strongly to all surfaces tested [17]. Clone 23C1, chosen as a non-selective antibody, was shown by immunofluorescence studies to concentrate at crystal imperfections, well known to be high energy binding sites. The nonspecific adsorption at imperfections may originate from general physical-attraction forces, such as may be generated by local induction of charges on the surface, or possibly from phenomena such as the self-aggregation of the antibody on a solid support.

Immunolabelling of cholesterol monohydrate crystals showed lower antibody coverage on the {001} face than on the {*h*0*l*} and {0*kl*} faces for all the monoclonal antibodies studied. The {001} faces are very smooth, whereas the {*h*0*l*} and {0*kl*} faces may present more imperfections. Labelling at imperfections would, however, result in a picture similar to that obtained for 23C1. We thus do not believe that the difference in labelling is due to differences in face topography, but rather to differences in structural and chemical composition. The (001) face of the cholesterol crystal has indeed a character very different from the other faces. In aqueous solutions, its surface layer is composed of lattice water molecules and of the hydroxyl groups of the cholesterol molecule. Lower affinity of proteins for surfaces covered by strongly bound lattice water molecules was similarly observed by Hanein *et al.* [22], using fibronectin as a test protein.

Among the selected monoclonal antibodies, MAb 58B1 displayed the highest degree of specificity for the cholesterol crystal surface, compared to all the other solid substrates studied. We observed that this antibody adsorbs onto the {*h*0*l*} faces of the crystal, rather than onto the {0*kl*} faces. On all the {*h*0*l*} faces, the cholesterol molecular backbone is exposed in the same orientation. Recognition for a given side of the crystal would tend to indicate that MAb 58B1 can recognize a repetitive motif comprising the part of the cholesterol molecule exposed in this direction. We cannot, however, exclude the possibility that preferred binding to a defined face of the (*h*0*l*) family occurs, but could not be detected under the conditions of our experiments. Indeed, with MAb 36A1, both immunolabelling techniques indicate a clear recognition for a given crystal face, either (301) or (401). These two faces are geometrically very close to the (100) face, towards which the antibody has a much lower affinity. The essential difference between the {301} and {100} faces is that the former are stepped faces, thereby exposing at the steps both the hydrophobic cholesterol backbones and the hydroxyl and water groups, a feature which could well be recognized by the antibody, whereas {100} faces expose only the cholesterol backbone (Fig. 8). At the molecular level the antibody might thus recognize a three-dimensional arrangement of molecules. Although {101} faces exhibit a similar stepped structure, the steps might be too close to each other to accommodate the bulky IgM molecule.

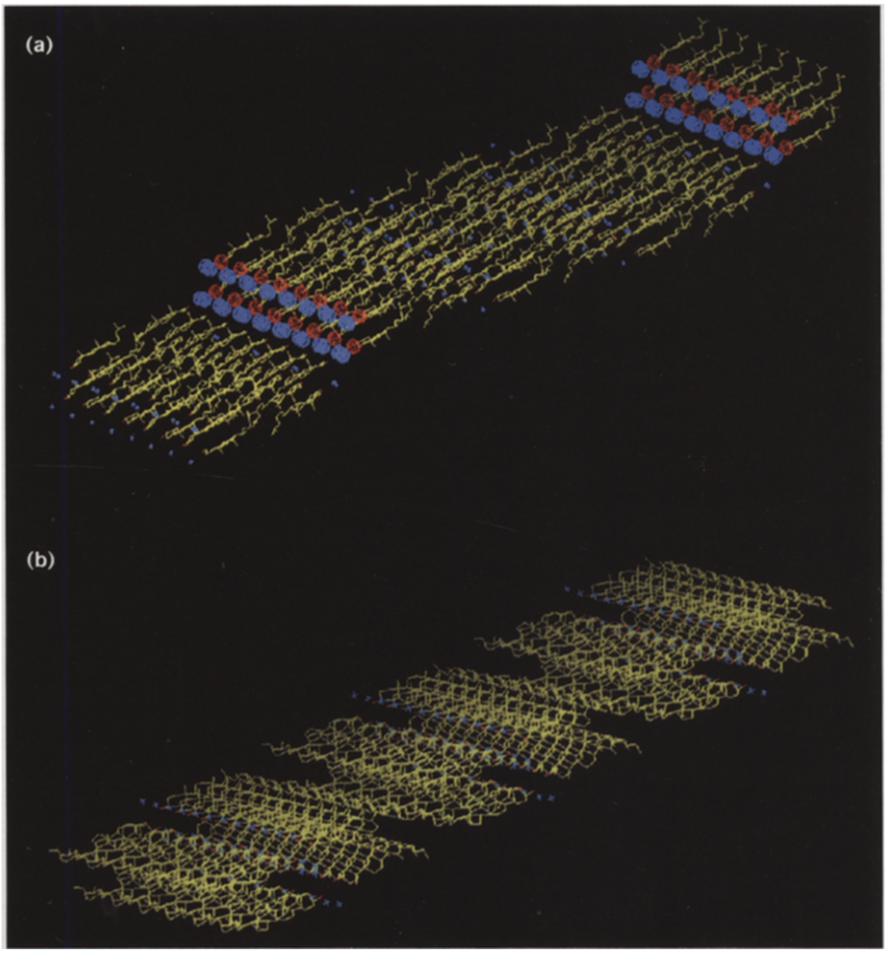
To obtain a more precise understanding of the recognition of an organized surface by a MAb, two types of studies have been initiated. First, by sequencing the antibody and modeling its structure in conjunction with the features recognized on the crystal faces, we hope to reach a molecular understanding of the interactions between the antibody and the crystal. We have also undertaken the synthesis of self-assembled monolayers exposing cholesterol entities in different orientations and configurations. The study of antibody recognition of these structured surfaces will permit a better definition of the surface parameters involved in the recognition process.

Significance

We have shown here that monoclonal antibodies directed against cholesterol crystal surfaces can be selected, and that they exhibit preferential recognition of some faces of the crystals relative to others.

Figure 8

The cholesterol and water molecules exposed at the (301) face and the (100) face differ. Packing arrangement of cholesterol monohydrate crystals on **(a)** the (301) face and **(b)** the (100) face. Yellow: cholesterol molecule backbone. Red: hydroxyl oxygen atoms. Blue: water molecules. The hydroxyl groups and water molecules exposed at the face are represented in solid, while those buried in the layer are represented by crosses.



Antibodies have evolved to be an almost infinitely tunable recognition tool for recognizing specific molecular structures. The immune system thus provides an ideal model for studying molecular recognition in biological macromolecules.

The fine details of the molecular interactions between antibodies and antigens are difficult to define systematically, however, because antigen structures vary over a wide range and are themselves not systematically defined. In contrast, crystal surfaces are homogeneous, repetitive, and present a controlled variability of structures that are atomically defined and whose organizations are known. A complete characterization of antibodies that recognize ordered structures such as crystals, and of their complexes with the specific selected faces, may thus assist in the understanding of antibody–antigen interactions in general. A detailed understanding of this kind may also provide insight into the immune response to extraneous solid surfaces inside the organism, which occur, for example, when crystalline structures form as part of the pathology of a disease or when foreign substances are surgically implanted.

Materials and methods

Materials

Cholesterol (99 % pure), cholesteryl hemisuccinate, 5-cholenic acid-3β-ol, 5-cholesten-3β-ol-7-one, *p*-nitrophenyl phosphate and bovine serum albumin (>98 % pure) were purchased from Sigma Chemical Co. (St. Louis, Missouri). Dimethylaminopropyl-N'-ethylcarbodiimidhydrochloride was purchased from Merck (Germany). C-carboxymethylamine hemihydrochloride was purchased from Aldrich (Milwaukee, WI). 1,4-Dinitrobenzene was purchased from Fluka (Switzerland). The antigen-binding fragments ($F(ab)_2$) of a goat-anti-mouse antibody, conjugated to alkaline phosphatase, rabbit-anti-mouse IgM and goat-anti-mouse $F(ab)_2$, rhodamine conjugated, were all obtained from Jackson Immunoresearch (West Grove, PA). Goat-anti-mouse IgG + IgM, conjugated with 30 nm colloidal gold was obtained from Bio-Cell (Cardiff, UK). Antibody 2C5-6 was obtained from the American Type Culture Collection (access number HB 8995). Spectra/por molecular porous membrane tubing was purchased from Spectrum Medical Industries, Inc. (Los Angeles, CA). Microtiter polystyrene plates were purchased from Nunclon (Denmark). Cholesterol crystals were grown on 13 mm microscope glass cover slips manufactured by Chance Propper Ltd. (Smethwick, Warley, England). Differential scanning calorimetry was performed with a Mettler TC1 TA processor. Scanning electron microscopy was performed with a Jeol JMS 6400. A Molecular Devices microplate reader was used for ELISA.

Growth of crystals
Cholesterol monohydrate was crystallized by heating 0.3 g cholesterol in 80 ml acetone to boiling, while stirring. Twenty ml of double-distilled water (DDW) were then added, and the solution was boiled for 30 s. Cooling to room temperature induces formation of a white cloudy suspension after a few hours. The solution was then shaken vigorously, and allowed to crystallize overnight. The crystals were filtered and used for ELISA experiments. The filtrate was further divided into 15-ml aliquots in 25-ml beakers, containing a cover glass slide. The crystals attached to the glass cover slides were used for immunolabelling experiments. The crystalline phases obtained were checked by differential scanning calorimetry [15] and the crystals examined under an optical microscope in white and cross-polarized light. Batches of crystals with a high level of imperfections were discarded. 1,4-DNB and 1,3-DNB crystals were grown as described [17].

Production of monoclonal antibodies
MAb 36A1 originated from a Balb/c mouse immunized with cholesterol monohydrate crystals by surgical implantation of the crystals in the spleen (twice at three-week intervals) followed by one intraperitoneal injection.

MAb 23C1, MAb 58B1 and MAb 122B1 were produced as described [17]. Screening was performed by ELISA on cholesterol and 1,4-DNB crystals in parallel. Isotyping was carried out using the mouse mono-AB Kit (HRP). Ascites were produced as described [23].

ELISA of antibody binding to crystals
For each sample, cholesterol monohydrate crystals (1.5 mg) were placed in a BSA-precoated eppendorf tube. Precoating was performed by incubation with 0.5 % BSA in phosphate-buffered saline (PBS), 1 h, followed by washing three times with PBS. All washings and separations were performed after spinning down the crystals by a short centrifugation. 200 µl of serum or hybridoma samples, sequentially diluted in binding buffer (PBS, 0.5 % BSA, 0.02 % NaN_3 saturated with cholesterol) were added, each dilution in duplicate. After incubation for 1 h at room temperature with gentle agitation, the crystals were washed twice with PBS saturated with cholesterol. The crystals were then incubated for 45 min with 200 µl alkaline-phosphatase-conjugated goat-anti-mouse $F(ab)'_2$, diluted 1/1000 in binding buffer, washed as previously, and incubated with 200 µl of substrate buffer (1 mg ml^{-1} of *p*-nitrophenylphosphate in 10 % diethanolamine buffer, 0.01 % $MgCl_2$, 0.02 % NaN_3) for 30 min, until the color developed. The reaction was stopped by addition of 50 µl 0.4 M EDTA, the eppendorf centrifuged, and the solutions transferred to microtiter plates. Absorption was determined at 402 nm. The same procedure was applied in parallel to BSA-coated eppendorf tubes without crystals, and the absorbance of the solution from the eppendorf without the crystals was subtracted from that of the samples.

For hybridoma screening the hybridoma medium, diluted 1/10, was incubated with the crystals in PBS saturated with cholesterol. After washing twice with PBS, 0.5 % BSA, 0.02 % NaN_3, the reaction was continued as described previously.

ELISA on 1,4-DNB crystals was performed in a similar manner, with the following modifications: i) 2 mg of crystals were used for each sample; ii) all the incubation and washing solutions were saturated with 1,4-DNB; iii) the crystals were transferred to a new BSA-coated eppendorf before adding the enzyme substrate, and the color was allowed to develop for 60 min.

Adsorption of monoclonal antibodies to polystyrene
The hybridoma culture medium was serially diluted in PBS, and adsorbed onto microtiter plates for 1 h, at room temperature. The plates were washed twice for 10 min each, with PBS/BSA, and blocked by incubation with PBS/BSA for 1h. The wells were incubated for 45 min with goat-anti-rabbit $F(ab)_2$, and washed three times for 5 min each with PBS, 1 % BSA. Color development was performed as described above with a 2-h incubation.

Cholesterol coating of polystyrene plates
Cholesterol was coated onto the surface of U-bottom wells, in microtiter plates, by addition of an ethanolic solution containing 1 or 10 µg cholesterol per well. As a control pure ethanol was added. The plate was dried and stored as described [15]. Blocking and reaction with monoclonal antibodies were carried out as described above. Blocking and reaction with ascites was performed as described [15].

Synthesis of 5-cholesten-3β-ol-7-(O-carboxymethyl)oxime
5-Cholesten-3β-ol-7-one (28 mg) was dissolved in ethanol (5 ml), and a solution of C-carboxymethylamine hemihydrochloride (17 mg) in 2N KOH (0.5 ml) was added. The mixture was refluxed for 3 h, cooled and concentrated by evaporation. Water (3 ml) was added, the mixture was extracted with ethylacetate (2 x 10 ml), and the product was purified by flash chromatography (7:3 ether:ethylacetate). ^1H NMR (Bruker 400 Mhz, $CDCl_3$) δ 0.7 (C-18 CH_3), 0.86 (C-26, CH3), 0.88 (C-27, CH_3), 0.93 (C-21, CH_3), 1.1 (C-19 CH_3), 1.2–2.5 (steroid backbone, 24H), 3.7 (C-3, H), 4.7 (O–CH_3–), 5.6 (C-4 H *anti*), 6.2 (C-4 H *syn*).

Conjugation of steroids to BSA
Cholesteryl hemisuccinate, 5-cholenic acid-3β-ol and 5-cholesten-3β-ol-7-(O-carboxymethyl)oxime were conjugated to BSA by activation of the carboxylic group using the following general procedure. Each steroid was dissolved in 1 ml well-dried dioxane (3×10^{-5} mole), 1.1 equivalent of 1,3-dicyclohexycarbodiimide and 1.1 equivalent of N-hydroxysuccinimide were added, and the solution was stirred for 24 h. The solution was filtered through a Pasteur pipette equipped with a cotton plug, and the resulting filtrate was combined with 0.015 equivalent BSA (dissolved in 2 ml PBS) and stirred overnight. The protein solution was placed in a cellulose dialysis membrane (SPECTRA/POR 6–8000 mw) and dialyzed against PBS (3 l) with five changes. The resulting dialysate was diluted with PBS, centrifuged and filtered through a 0.2 µm filter.

Alternatively, BSA conjugates were prepared by direct attachment via N-(3-dimethylaminopropyl)-N'-ethylcarbodiimidhydrochloride (EDC). A solution of BSA (20 mg), steroid (5 mg) and EDC (3 mg) was prepared in 10:1 DDW:pyridine. The mixture was stirred overnight. The protein solution was placed in a cellulose dialysis membrane and dialyzed against DDW (3 l) with three changes. The resulting dialysate was diluted with deionized water, centrifuged and filtered through a 0.2 µm filter.

Characterization of the BSA–steroid conjugates
The BSA–steroid conjugates were characterized by hydrolysis of the steroids and measurement of their concentration by NMR. Each BSA–steroid sample (4 ml) was lyophilized, ethanol (5 ml) and KOH (300 µl, 33 % v/v) were added, and the solution stirred for 24 h at 35 °C. The hydrolyzed solution was extracted with water (10 ml) and chloroform (10 ml), the organic layer was separated and evaporated to dryness. The amount of steroid was quantified by NMR spectroscopy using dioxane as an internal standard and comparing the intensity of the methyl peaks to that of the standard.

Binding of the monoclonal antibodies to protein conjugates
Protein conjugates were diluted 1/10 and coated onto 96-well microtiter plates, by addition of 100 µl of protein solution to each well. The plate was incubated for 2 h, washed twice with PBS and kept overnight at 4 °C. The wells were blocked by incubation with 0.25 % gelatin in PBS for 30 min, followed by two washes with PBS. Antibody ascites (36A1, 58B1, 122B1 and 2C5-6), diluted 1/10 in 0.25 % gelatin in PBS, were incubated for 1 h, at room temperature. The plates were washed three times for 5 min each with PBS, incubated with goat-anti-mouse $F(ab)_2$ secondary antibody and again washed three times for 5 min each with PBS. Color development was performed as described above.

Immunofluorescence and immunogold labelling experiments
Cholesterol monohydrate crystals, grown attached to glass cover slides (1.3 cm), were placed in 24-well polystyrene dishes precoated

with BSA. The crystals were incubated 1 h with hybridoma media diluted 1/20 in binding buffer, at room temperature, with slow agitation. They were washed three times for 5 min each with washing buffer, and incubated 1 h with rhodamine-labelled goat-anti-mouse $F(ab)_2$ antibody diluted 1/100, or 30 min with 30 nm colloidal gold-conjugated goat-anti-mouse IgG + IgM antibody, diluted 1/50. The crystals were washed again with washing buffer as above, and very briefly with DDW. The fluorescently labelled crystals were transferred to microscope slides, air dried and observed under fluorescent light with an optical microscope. The cover slides with the gold-labelled crystals were air dried, frozen in liquid nitrogen, lyophilized overnight, sputter-coated with gold, and examined in the scanning electron microscope at 15 kV by secondary electron emission. Lyophilization of cholesterol monohydrate crystals prevents damage, because it prevents lattice water from being extracted from the crystals by the electron beam or by the sputtering process. However the lyophilized samples are only stable for up to 72 h. Beyond this time period the crystals presumably undergo a phase transition and disintegrate.

Cholesterol monohydrate crystals grown under the conditions of our experiment expressed well developed {*h*0*l*} and {0*kl*} faces. The Miller indexes of the faces were determined by accurately measuring the dihedral angle between these faces and the easily identifiable (001) plane of the crystals, from electron micrographs taken with the faces exactly edge-on to the plane of the picture. This identifies unambiguously whether the crystal is being examined down *a**, ((*h*0*l*) faces) or down *b**, ((0*kl*) faces). To identify the faces viewed face-on to the picture, the crystals were tilted up to 80°, and the angle between the (001) plane and the faces examined was assessed, as close as possible to an edge-on position. Once the faces were identified, the gold beads were counted in at least three different locations on each face, at magnification 20 000x. Only samples where the distribution and the number of gold beads could be unambiguously established were taken into account.

Labelling of cholesterol crystals with dansylated BSA

Labelling of BSA with dansyl chloride was performed by mixing 200 μl of a solution of 2.5 mg dansyl chloride per ml acetone with 200 μl of 0.2 M $NaCO_3$, pH 8.5, containing 5 mg BSA. The mixture was stirred 2 h at room temperature, and dialyzed three times against 500 ml PBS. Cholesterol monohydrate crystals were incubated 2 h in PBS saturated with cholesterol, containing 0.01 % dansyl-BSA, with or without 0.5 % unlabelled BSA. The crystals were then washed three times with PBS saturated with cholesterol, and examined under fluorescent light with an optical microscope.

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