

Novel anti-cholesterol monoclonal immunoglobulin G antibodies as probes and potential modulators of membrane raft-dependent immune functions

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Abstract Natural autoantibodies against cholesterol are present in the sera of all healthy individuals; their function, production, and regulation, however, are still unclear. Here, we managed to produce two monoclonal anti-cholesterol antibodies (ACHAs) by immunizing mice with cholesterol-rich liposomes. The new ACHAs were specific to cholesterol and to some structurally closely related 3 β -hydroxyl sterols, and they reacted with human lipoproteins VLDL, LDL, and HDL. They bound, usually with low avidity, to live human or murine lymphocyte and monocyte-macrophage cell lines, which was enhanced substantially by a moderate papain digestion of the cell surface, removing some protruding extracellular protein domains. Cell-bound ACHAs strongly colocalized with markers of cholesterol-rich lipid rafts and caveolae at the cell surface and intracellularly with markers of the endoplasmic reticulum and Golgi complex. These data suggest that these IgG ACHAs may serve as probes of clustered cholesterol (e.g., different lipid rafts) in live cells and thus may also have immunomodulatory potential.—Bíró, A., L. Cervenak, A. Balogh, A. Lőrincz, K. Uray, A. Horváth, L. Romics, J. Matkó, G. Füst, and G. László. Novel anti-cholesterol monoclonal immunoglobulin G antibodies as probes and potential modulators of membrane raft-dependent immune functions. *J. Lipid Res.* 2007. 48: 19–29.

Supplementary key words membrane cholesterol • lymphocytes • monocyte-macrophage cells • lipoproteins • lipid rafts • caveolae

Because of its widespread distribution and important biological roles, cholesterol has long been assumed to be a nonimmunogenic or poorly immunogenic molecule. However, several laboratories reported the activation of the classical and alternative pathways of the complement sys-

tem by cholesterol (1–4). Natural autoantibodies of both IgM and IgG isotypes against cholesterol were shown to be present ubiquitously in the sera of healthy individuals (2, 3). Because cholesterol is an evolutionally conserved, abundant structural lipid in the plasma membrane of mammalian cells, it was tempting to speculate whether naturally occurring antibodies against cholesterol could bind to the surface of cells. Under physiological conditions, antibodies against lipids, such as phospholipids or cholesterol, are not expected to bind with high affinity to intact cell membranes, because of the small size of the antigen epitope and the steric hindrance by surrounding glycolipids or large, protruding glycoproteins (3). However, when either the affinity of anti-lipid antibodies or the molecular pattern/morphology of the cell surface is altered, such interaction between antibodies and lipids may take place (e.g., under pathological conditions).

Monoclonal anti-cholesterol antibodies (ACHAs) were first reported in 1988 by Swartz et al. (1) after immunizing mice with cholesterol-containing liposomes, together with lipid A as adjuvant. They found cholesterol to be a surprisingly excellent immunogen, and murine monoclonal IgM-type complement-fixing antibodies to cholesterol were easily obtained (1). Perl-Treves et al. (4) developed and examined monoclonal IgM antibodies against cholesterol monohydrate crystals by implantation of cholesterol crystals in the spleen of mice. This choice was motivated by the fact that cholesterol, in the living organism, may aggregate or precipitate in pathological situations as microcrystals (gallstones, atherosclerotic plaques). These IgM antibodies did not seem to recognize the cholesterol molecule itself but rather a de-

Manuscript received 5 April 2006 and in revised form 11 August 2006 and in re-revised form 3 October 2006.

Published, *JLR Papers in Press*, October 5, 2006.
DOI 10.1194/jlr.M600158-JLR200

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ASBMB
JOURNAL OF LIPID RESEARCH

fined, extended pattern of molecular motifs exposed on the cholesterol crystal surfaces. Kruth et al. (5) directly visualized plasma membrane cholesterol clusters with a monoclonal antibody that specifically detects ordered cholesterol arrays, but only when the cells were previously enriched with cholesterol. This antibody detected a specific plasma membrane pool of cholesterol, responsive to agents that modulate cholesterol trafficking. The cholesterol-rich domains they detected in the plasma membrane did not seem equivalent to the so-called lipid rafts (6), because they were highly sensitive to ice-cold Triton X-100 extraction, whereas lipid rafts are not.

The presence of ACHAs in normal serum raised another important question: do these antibodies against cholesterol play a role in the regulation of cholesterol levels, and by extension, in atherosclerosis, *in vivo*? A putative role of ACHAs in atherosclerosis, induced by a cholesterol-rich diet, can be postulated from results of experiments with rabbit models (7).

Altered serum levels of ACHA were reported in patients with atherosclerosis (8, 9) and with chronic viral infections (8, 10). Although IgG isotype ACHAs can be detected in the sera of both healthy individuals and patients with several diseases, only IgM isotype ACHA has been produced and characterized to date. Thus, the question emerged whether it is possible to produce IgG isotype monoclonal antibody against cholesterol by immunization. From a practical point of view, an IgG isotype antibody would be much more convenient in most immunological techniques, such as immunohistochemistry, flow cytometry, and ELISA.

Furthermore, generation and *in vitro* characterization of the biological activity of IgG-type ACHAs seem critically important steps toward understanding their physiological roles. On the other hand, various cholesterol- and sphingolipid-rich membrane microdomains (e.g., the caveolar and noncaveolar lipid rafts) were implicated in controlling/regulating numerous cellular functions (6, 11–15), including activatory and inhibitory signal transduction events and the cellular uptake of various pathogens (viruses, bacteria, protozoa) and toxins (16, 17). Thus, IgG-type ACHAs might have significance in probing or functional modulation of such membrane microdomains.

Therefore, the aim of this study was to produce IgG isotype monoclonal ACHAs by immunizing mice with cholesterol-enriched liposomes. We managed to produce two IgG isotype monoclonal antibodies against cholesterol (AC1 and AC8) and found that they specifically detect cholesterol and several structurally closely related sterols in cell-free assays, as well as specifically bind to human lipoprotein fractions. The IgG-type ACHAs also bound to various cellular compartments, both extracellularly and intracellularly, identified as clustered plasma membrane cholesterol, lipid rafts, caveolae or intracellular lipid vesicle membranes, endoplasmic reticulum, and Golgi compartments. The basic properties, selectivity, and cellular and subcellular localization of the novel IgG3 ACHAs,

as well as their potential application areas, are described and discussed.

MATERIALS AND METHODS

Cells

Jurkat [American Type Culture Collection (ATCC)] and MT-4 (HTLV-1+; a generous gift from Z. Beck, University of Debrecen, Hungary) human T-cell lymphomas and MonoMac6 (a kind gift of Z. Bajtay, Eötvös University, Budapest) and U937 (ATCC) human monocyte-macrophage cell lines were cultured in RPMI 1640 supplemented with 10% fetal calf serum. Murine P388D1 and J774A.1 monocyte-macrophage cell lines, A20 (ATCC) B lymphoma cells, and the Keyhole Limpet Haemocyanin-specific murine T_H hybridoma (2/13), produced as described previously (18), were all cultured in RPMI 1640 supplemented with 10% fetal calf serum and 2-mercaptoethanol.

Cholesterol liposomes

Cholesterol-rich multilamellar vesicles (71 mol% cholesterol) used for the induction of antibody production to cholesterol were composed as described by Dijkstra et al. (19). Briefly, they were made of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol, and cholesterol in a molar ratio of 9:1:25, and for immunization they were completed with adjuvant monophosphoryl lipid A at 25 µg/µmol phospholipid. Reagents were purchased from Sigma-Aldrich (St. Louis, MO). The lipids were divided into aliquots from stock solutions in chloroform in a pyrogen-free, round-bottom flask, and the solvent was removed by rotary evaporation. Sterile deionized water was added to the dry lipid film, achieving 50 mM total phospholipid, and vortexed until all of the lipids were resuspended. The material was subsequently incubated for 2 h at room temperature. Aliquots of the liposomal preparations were lyophilized in vaccine vials, stored at -70°C, and reconstructed before use in PBS to 10 mM final concentration of total phospholipids.

Immunization with cholesterol-rich liposomes

For the induction of antibodies to cholesterol, four male inbred mice (BALB/c) were immunized with 100 µl of the cholesterol-rich multilamellar vesicles (2.5 µmol of cholesterol) intraperitoneally and subcutaneously. After 4 weeks, the mice were given a boost injection of 2.5 µmol of cholesterol. Two weeks later, the mice were bled from the tail vein to collect immune sera. Preimmune sera were collected just before the primary inoculation; a 1:50 dilution was used to measure reactivity. ELISA for cholesterol was used to determine serum titer change in mice, and one of them was selected to be used for cell fusion. The animal studies were reviewed and approved by the appropriate institutional review committee.

Production of monoclonal antibodies against cholesterol

Monoclonal antibodies were generated by hybridizing Sp2/0-Ag14 myeloma cells with the splenocytes of immunized mice. Hybridomas were selected by growth in hypoxanthine-aminopterin thymidine medium. Initial screening was performed by ELISA on cholesterol. Two IgG and eight IgM clones were selected for expansion and initial characterization. Isotyping was carried out by anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM, κ, and λ horseradish peroxidase-conjugated antibodies (SouthernBiotech, Birmingham, AL).

The two IgG3 subclones, AC1 and AC8, were further characterized in detail. The IgGs were purified on Protein-G Sepharose

4 Fast Flow (Amersham Pharmacia Biotech AB, Uppsala, Sweden) columns by affinity chromatography from the supernatant of the hybridoma cells. The purity of antibodies was analyzed by SDS-PAGE.

Enzyme-linked immunoassay of antibody binding to cholesterol and other lipids

Polystyrene plates (Greiner, Frickhausen, Germany) were coated with cholesterol (0.01–10 µg/well), ergosterol, coprostane, 7-ketocholesterol, cholesteryl oleate, 25-hydroxy-cholesterol, cholic acid, DMPC, DL-alpha-dipalmitoyl-phosphatidylcholine (DPPC), or L-alpha-dipalmitoyl-phosphatidylethanolamine (DPPE) (10 µg/well for all lipids) (Sigma) dissolved in 100 µl of absolute ethanol and incubated at 4°C for 24 h. After washing with PBS and blocking with 0.1% casein (Reanal, Budapest, Hungary) in PBS, wells were incubated with 100 µl of serum samples or purified IgG diluted in PBS containing 0.1% casein. Binding of ACHAs was detected by anti-mouse horseradish peroxidase-conjugated γ -chain-specific goat antibodies (DAKO, Glostrup, Denmark), with *o*-phenylenediamine (Sigma) and H₂O₂ as substrates. The optical density was measured at 490 nm (reference at 600 nm). Concentrations of monoclonal antibodies at which an optical density of 0.2 (490–600 nm) was reached are demonstrated by the following symbols in **Table 1**: –, no reaction (at 100 µg/ml); +, low reaction (10–100 µg/ml); ++, medium reaction (1–10 µg/ml); +++, strong reaction (<1 µg/ml).

Competitive ELISA with human lipoproteins

Purified monoclonal IgG was mixed with different concentrations of human lipoproteins VLDL, LDL, and HDL (Calbiochem, San Diego, CA), or with 0.1% casein as a control, and incubated at 37°C for 60 min. The preincubated mixtures were applied to cholesterol-coated plates, and IgG binding was detected as described previously. Lipoprotein concentrations were normalized to the same surface area using the following data. For particle mass: VLDL, 40,000 kDa/mol; LDL, 2,300 kDa/mol; HDL, 200 kDa/mol. For particle diameter: VLDL, 50 nm; LDL, 20 nm; HDL, 10 nm. Initial concentrations used for the serial dilutions

TABLE 1. Cross-reactivity of monoclonal anti-cholesterol IgG3 antibodies AC1 and AC8 with other lipids

Lipid Type	Name	AC1	AC8
Sterols containing free 3 β -hydroxyl	Cholesterol	++	+++
	Ergosterol	+	+++
	7-Ketocholesterol	++	+++
	25-Hydroxy-cholesterol	–	–
Sterols lacking free 3 β -hydroxyl	Cholesteryl oleate	–	–
	Coprostane	–	–
	Cholic acid	–	–
Nonsterol lipids	1,2-Dimyristoyl- <i>sn</i> -glycero-3-phosphocholine	–	–
	DPPC	–	–
	DPPE	–	–
	1,2-Dimyristoyl- <i>sn</i> -glycero-3-phosphoglycerol	–	–

DPPC, DL-alpha-dipalmitoyl-phosphatidylcholine; DPPE, L-alpha-dipalmitoyl-phosphatidylethanolamine. ELISA plates were coated with cholesterol, ergosterol, 7-ketocholesterol, 25-hydroxy-cholesterol, cholesteryl oleate, coprostane, cholic acid, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol, DPPC, or DPPE. AC1 and AC8 antibodies were serially diluted and added to the plates. Concentrations of monoclonal antibodies at which an optical density of 0.2 (490–600 nm) was reached are demonstrated by the following symbols: –, no reaction (at 100 µg/ml); +, low reaction (10–100 µg/ml); ++, medium reaction (1–10 µg/ml); +++, strong reaction (<1 µg/ml).

were determined based on normalization for the surface area corresponding to 20 µg of HDL.

Measurement of fluorescence resonance energy transfer

2-(4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phospho-choline (BODIPY-FL C₅-HPC) was purchased from Molecular Probes-Invitrogen (Eugene, OR). Fluorescence resonance energy transfer (FRET) from BODIPY-FL C₅-HPC (donor) bound to HDL and LDL to Alexa555-conjugated AC8 antibody (acceptor) was measured by recording the emission spectra of BODIPY-FL C₅-HPC-labeled lipoproteins in the absence or presence of fluorescent AC8 on an Edinburgh Instruments F900 fluorescence spectrometer (Livingston, UK), using 460 nm excitation wavelength. Anti-human CD11a/LFA-1 α antibody (MEM-25; a kind gift from Vaclav Horejsi, Prague, Czech Republic) was used as a negative control. The efficiency of FRET was estimated from both the quenching of donor fluorescence and the sensitization of acceptor fluorescence (20).

Detection of cell-bound ACHA by flow cytometry and confocal microscopy

The lymphoid and human monocyte-macrophage cell lines were incubated with 1–10 µg/ml monoclonal ACHA for 30 min on ice, washed and incubated with Alexa488-conjugated goat anti-mouse IgG secondary antibody (Molecular Probes-Invitrogen), and assayed by flow cytometry. Alternatively, directly labeled Alexa488-ACHA was also used to label cells for flow cytometry or confocal laser scanning microscopy, using Ka40 tobacco mosaic virus-specific mouse monoclonal antibody (produced in our laboratory) as an isotype control. For intracellular staining, cells were incubated with Alexa488-AC8 after fixation by 2% formaldehyde and permeabilization by 0.1% saponin plus 0.1% BSA. Cells (5 × 10⁵ cells/sample) were labeled for all experiments. In flow cytometric experiments, 10,000 cells were collected and analyzed in a Becton-Dickinson FACSCalibur flow cytometer using CellQuest Pro software. In confocal laser scanning microscopy imaging experiments, the labeled cells were placed onto 0.15 mm thin coverslips in a chamber and assayed with an Olympus (Hamburg, Germany) Fluoview500 confocal microscope equipped with four optical channels using a 60 \times , high-numerical aperture oil-immersion objective.

Papain treatment of cells

Cells were exposed to a limited digestion by 5 or 10 U/ml papain (Sigma) with 10 U/mg specific activity for 5 and 10 min at 37°C in HBSS (Gibco). Cells were then washed extensively with ice-cold FACS buffer (Becton-Dickinson) supplemented with 0.1% BSA and labeled with antibodies as described previously.

Modification of membrane cholesterol level/distribution

Membrane cholesterol level was modulated by depletion with methyl- β -cyclodextrin (RAMEB, CycloLab), treating the cells with 2–10 mM methyl- β -cyclodextrin for 15 min at 37°C, as described previously (21), or with cholesterol oxidase (Sigma) (22). Briefly, cells were incubated with 0.5 or 1 U/ml cholesterol oxidase (from *Pseudomonas fluorescens*) for 2 h at 37°C and washed extensively. For complexing and segregating membrane cholesterol, 1–10 µg/ml concentrations of filipin III antibiotics (Sigma) were applied for a 45 min incubation at 37°C (23).

Isolation of detergent-insoluble buoyant membrane from T_H cells and immunodot blot analysis

Detergent-resistant and -soluble membrane fractions were isolated from T_H cells by a cold Triton X-100 lysis and subsequent

separation using sucrose density gradient (5–40%) ultracentrifugation, as described previously (24). Immunodot blots were developed on nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Cholesterol was diluted from stock solution in absolute ethanol to 50 $\mu\text{g}/\text{ml}$ using PBS, and detergent-resistant (low buoyant density: 5% sucrose) and detergent-soluble (high buoyant density: 36–40% sucrose) membrane fractions were dropped onto the membrane in small amounts (2 μl) either directly or at 2 \times dilution and dried. Pretreatment of the samples with cholesterol oxidase was performed as described above. After blocking with 0.1% casein in PBS for 1 h, AC8 was added (1.5 $\mu\text{g}/\text{ml}$) for 1 h and then washed three times with PBS. The dot blots were finally developed by incubating with horseradish peroxidase-conjugated GAM IgG3 (SouthernBiotech) for 1 h, followed by washing six times with PBS, and evaluated using enhanced chemiluminescence reagent (Pierce Chemicals, Rockford, IL) and X-ray film (Agfa, Mortsel, Belgium).

Confocal microscopic colocalization assay

Cells were stained for lipid raft markers (GM1 gangliosides) with Alexa488- or Alexa647-cholera toxin B (Molecular Probes-Invitrogen), according to the manufacturer's instructions; FITC-anti-caveolin-1 antibody (rabbit IgG; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used to label caveolae after a mild permeabilization of the plasma membrane (25). The long-chain diIC18 (3) (Molecular Probes-Invitrogen) lipid probe partitioning into liquid-ordered/gel-phase membrane regions was used according to the manufacturer's instructions. Human CD71-specific Cy3-MEM-75 (mouse IgG1 antibody) was a kind gift from Vaclav Horejsi, and FITC-conjugated rat anti-mouse antibody G7.4 (IgG2c; from ATCC) was used to label Thy1. CD2 on T-cells were labeled with FITC-conjugated anti-human CD2 monoclonal antibody (Sigma). ACHAs were applied either as Alexa488-conjugated AC8 or biotinylated AC8 plus Alexa647-conjugated neutravidin. For all antibodies, appropriate isotype control antibodies or cell lines were used as negative controls. BODIPY-brefeldin A558 (fixed/permeabilized cells, 100 nM, 30 min, 37°C) and BODIPY FL C₅-ceramide (live cells, 5 μM , 30 min, 4°C; Molecular Probes-Invitrogen) were used as markers of the endoplasmic reticulum/Golgi complex, whereas LysoTracker 568 (live cells, 75 nM, 30 min, 37°C; Molecular Probes-Invitrogen) served as a marker of lysosomes. Cell nuclei and mitochondria were stained with Draq5 (live cells, 2.5 μM , 10 min, room temperature; Biosstatus, Ltd., Leicestershire, UK) and MitoTracker (live cells, 50 nM, 30 min, 37°C; Molecular Probes-Invitrogen), respectively. All of these markers were applied for cell labeling as described in the manufacturer's protocol. For plasma membrane cholesterol staining, live cells were used; to identify the subcellular localization of cholesterol, however, cells were fixed/permeabilized, as described above. Colocalization indices were determined with ImageJ software using appropriate plug-ins (Image Correlator Plus), from >100 regions of interest per sample, selected from at least 20 cells.

Statistical analysis and image processing

GraphPad Prism 4 for Windows (version 4.02) software (GraphPad Software, San Diego, CA) was used for the statistical analyses. Inhibitory effects of lipoproteins were compared by two-way ANOVA. Confocal microscopic images were further processed and analyzed with ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD), available at the website of the Wright Cell Imaging Facility (Toronto, Ontario, Canada; www.uhnres.utoronto.ca/facilities/wcif.htm).

Monoclonal antibody production

Preimmune sera reacted with cholesterol very weakly, similar to healthy mouse control sera (Sigma). After immunizing with liposomes containing 71% cholesterol, both IgG and IgM ACHA titers were largely increased (data not shown). Monoclonal antibodies were generated by hybridizing Sp2/0-Ag14 myeloma cells with spleen cells from immunized mice of the highest antibody titer. After subcloning, three IgG- and eight IgM-secreting hybridomas were selected based on their capacity to produce antibodies reacting with cholesterol. All of the IgG antibodies were of the IgG3 isotype, and all of the IgG and IgM antibodies had κ light chains. Several IgMs specific to cholesterol had already been generated and characterized (1, 4, 19); therefore, we focused our investigations on the IgGs. Two selected clones (AC1 and AC8) were further analyzed in terms of their specificity and binding to intact cells (plasma membrane/subcellular localization).

Reactivity of monoclonal IgG ACHAs to cholesterol and other lipids

When applied in solid phase to ELISA plates, cholesterol has been reported to show a concentration-dependent aggregation (4). At 1 μg of cholesterol per well, cholesterol dominantly exists in noncrystalline form, except for a few microscopic crystals, whereas a significant amount of microcrystals exists at 10 μg of cholesterol per well. To determine whether the clustering or crystal structure of cholesterol has an effect on the binding of our monoclonal antibodies, plates were coated with 0.01–10 $\mu\text{g}/\text{well}$ cholesterol, and ACHA binding was tested in an ELISA system. Both AC1 and AC8 monoclonal antibodies bound in a dose-dependent manner to cholesterol adsorbed on polystyrene plates. The binding of both monoclonal antibodies was fairly independent of cholesterol concentration between 0.1 and 10 $\mu\text{g}/\text{well}$ cholesterol. Their binding capacity, however, declined sharply to <0.1 $\mu\text{g}/\text{well}$ (Fig. 1). The isotype control antibody (Ka40) did not bind to the coat (Fig. 1B).

The binding of ACHAs to various structurally related sterols and to other lipids was also tested by ELISA. The 3 β -hydroxyl group was proposed previously as a critical structural motif in the binding of anti-cholesterol IgMs (9). To investigate the fine specificities of AC1 and AC8, six structurally related sterols (ergosterol, coprostanone, cholesteryl oleate, 25-hydroxy-cholesterol, 7-ketocholesterol, and cholic acid) and three other nonsterol lipids (DMPC, DPPC, and DPPE) were tested as coated antigens. Like cholesterol, ergosterol, 7-ketocholesterol, and 25-hydroxy-cholesterol contain a free 3 β -hydroxyl group, whereas cholic acid, coprostanone, and cholesteryl oleate do not. ELISA results showed a more efficient binding of AC8 to cholesterol, ergosterol, and 7-ketocholesterol than to the other sterols. AC1 consistently showed a lower affinity to all sterols than did AC8, and the sterols to which they bound with the highest affinity were different (cholesterol for AC1 and 7-ketocholesterol for AC8). This indicates that the molec-

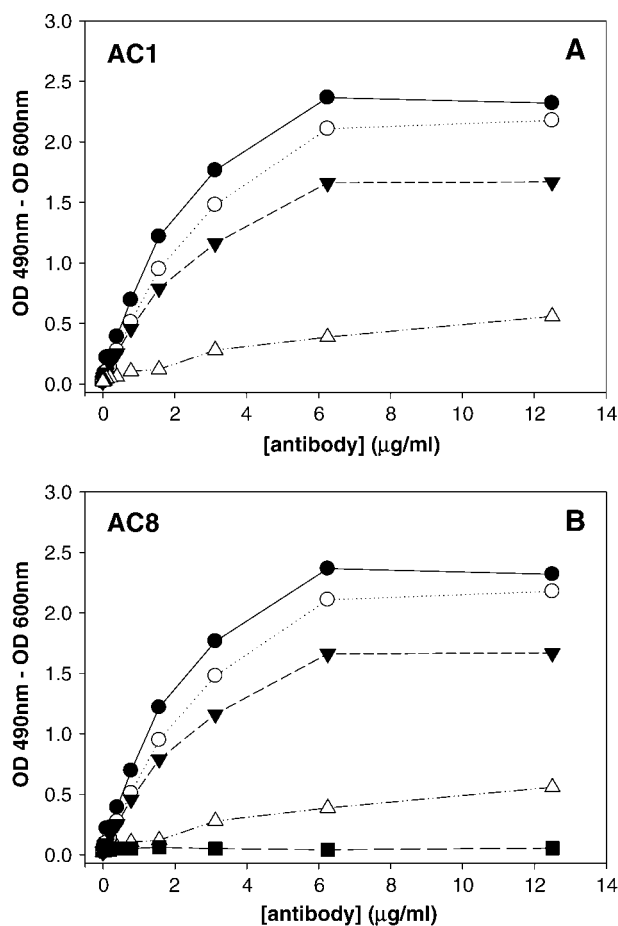


Fig. 1. Binding of monoclonal antibodies to cholesterol. Cholesterol was adsorbed to polystyrene plates at concentrations of 0.01 (open triangles), 0.1 (closed inverted triangles), 1.0 (open circles), and 10 (closed circles) $\mu\text{g}/\text{well}$. ELISA was carried out with serially diluted purified monoclonal IgG [initial concentrations: AC1 IgG, 100 $\mu\text{g}/\text{ml}$ (A); AC8 IgG, 25 $\mu\text{g}/\text{ml}$ (B)], then goat anti-mouse IgG labeled with horseradish peroxidase was applied. In B, binding of the isotype control Ka40 antibody (closed squares) is also displayed. Representative results from three independent experiments are shown. OD, optical density.

ular determinants may be slightly different for AC8 and AC1. In spite of the presence of a 3β -hydroxyl group of 25-hydroxy-cholesterol, the ACHAs did not bind to it. On the other hand, there was no detectable binding to other sterols lacking the 3β -hydroxyl group (Table 1). These results together suggest that the epitope recognized by ACHAs comprises the 3β -hydroxyl group in sterols, although other parts of the structure (not present in 25-hydroxy-cholesterol) cannot be excluded. As a control, no significant binding of IgG3 ACHAs to three other selected phospholipids was observed (Table 1).

The IgG3 ACHAs specifically bind to lipoproteins

Lipoproteins contain a significant amount of cholesterol in both the free and esterified forms. To assess the ability of ACHAs to interact with the human lipoproteins VLDL, LDL, and HDL, purified ACHAs were premixed with different concentrations of lipoproteins. After incu-

bation at 37°C for 60 min, the mixture was applied to cholesterol-coated plates. All of the human lipoproteins (VLDL, LDL, and HDL) inhibited the binding of ACHAs to cholesterol in a dose-dependent manner (Fig. 2A). The lipoproteins did not bind to the cholesterol coat under the same conditions (data not shown). When the results were normalized to the surface areas (corresponding to the available binding sites for antibody) of the various lipoproteins, binding of ACHA to HDL was found to be only

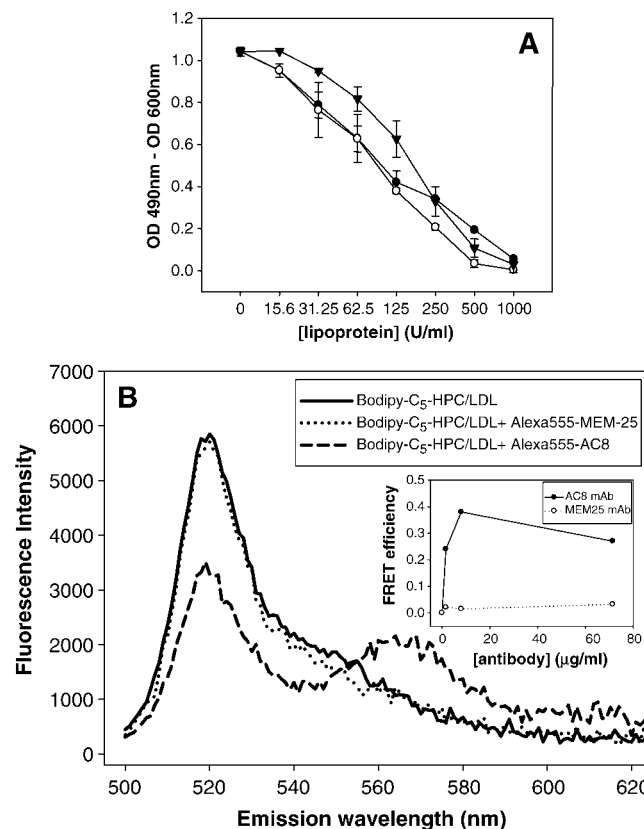


Fig. 2. Binding of anti-cholesterol IgG3 antibodies (ACHAs) to lipoproteins. A: Inhibition of the ACHA-cholesterol interaction by human lipoproteins. Serially diluted liposomes or human VLDL (closed circles), LDL (open circles), and HDL (closed inverted triangles) were mixed with anti-cholesterol IgG, AC8 (10 $\mu\text{g}/\text{ml}$). The surface of 20 μg is equal to $1.8 \times 10^{-2} \text{ m}^2$. All the concentrations of lipoproteins (HDL, LDL, VLDL) were adjusted to this surface. The mixture was incubated at 37°C for 60 min, then dropped onto cholesterol-coated ELISA plates. Bound IgG was detected by goat anti-mouse IgG labeled with horseradish peroxidase. Means of three independent experiments \pm SEM are shown. No significant differences were found between the inhibitory effects of the three lipoproteins using two-way ANOVA. OD, optical density. B: In fluorescence resonance energy transfer (FRET) measurements, LDL (500 μg total lipid/ml) with or without 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phospho-choline (BODIPY-FL C₅-HPC; 110 nM) was titrated with increasing concentrations (X axis) of Alexa555-AC8 or Alexa555-anti-CD11a (as a negative control). FRET efficiencies (see inset) with AC8 (solid line) or anti-CD11a (dotted line) acceptors were calculated from either the decrease of corrected donor emission intensities at 519 nm or the acceptor sensitization at 570 nm, upon excitation at 460 nm. mAb, monoclonal antibody.

slightly weaker than to VLDL or LDL, in contrast to earlier findings with IgM ACHAs. Binding of AC8 to lipoproteins (LDL and HDL) was further confirmed by direct FRET measurements between BODIPYFL C₅-HPC lipid probe and Alexa555-AC8. A highly efficient ($E_{\max} \sim 0.4$) and concentration-dependent FRET was observed in LDL (Fig. 2B). Similar, but less efficient ($E_{\max} \sim 0.3$), FRET was obtained with HDL (data not shown). No FRET was detected when Alexa555-anti-CD11a antibody was used as the acceptor.

IgG3 ACHAs bind to various intact cells both extracellularly and intracellularly

Several cell types of different origin, expressing lipid rafts or caveola microdomains enriched in cholesterol, were selected to investigate the binding of monoclonal ACHAs to the cell surface or to intracellular compartments: mouse helper T-cell hybridoma (2/13), human T-lymphoma cell lines (Jurkat, MT-4), human monocyte-macrophage cell lines (MonoMac6, U937), and murine B- and macrophage cell lines (A20 and P388D1, respectively). A low-avidity spontaneous binding of both IgG3 ACHAs was observed in all cell lines, which was cell type-dependent (Fig. 3A–D). The expression pattern of long, protruding glycoproteins or glycolipids, which may shield the presumably small antigen determinant(s) of ACHAs, may vary among cell types. Therefore, we tested whether a limited papain digestion of the cell surface, expected to remove some long external protein domains, affects the binding of our ACHAs. Indeed, an optimized papain treatment significantly decreased the magnitude of immunofluorescence arising from several long cell surface glycoproteins, such as CD45R/B220 on B-cells and CD44 on T-lymphocytes (Fig. 3E, F). Binding of AC8 antibody substantially increased in all cell types upon papain digestion, proportionally with the papain dose (Fig. 3A–D), and the differences in the extent of ACHA binding among cell types also became more pronounced. This suggests that a limited digestion of the cells by papain (10 U/ml, 10 min, 37°C) may serve as an “epitope exposition tool” for ACHAs on either fixed or unfixed cells of different origin, allowing “snapshot imaging” of clustered cholesterol at the surface of cells under various physiological or pathological conditions (Fig. 4).

Confocal microscopy showed that AC8 IgG intensely stained the plasma membrane of murine T_H cells after papain digestion (Fig. 4B, C), whereas no or very weak staining was observed with the fluorescent isotype control antibody Ka40 (Fig. 4A) or in the absence of epitope exposition by papain (data not shown), respectively, in accordance with the flow cytometric data (Fig. 3A–D). The plasma membranes of various cells were labeled by AC8 uniformly, in a strongly patchy manner, with an approximate average patch size of 200–400 nm (Fig. 4C).

Membrane ganglioside (raft) expression, assessed by flow cytometric analysis of fluorescent cholera toxin B binding, showed a fairly good correlation with the magnitude of ACHA binding on various murine and human cell types (Fig. 3G). Notably, monocyte-macrophage cells were represented mostly in the high double expression region (Fig. 3G, right side), possibly because of their ca-

veolar raft expression. In contrast, an IgM isotype ACHA, displaying cholesterol selectivity in cell-free assays, did not show detectable binding to any of the tested cell types, even after papain digestion (data not shown).

AC8 antibody also bound intracellularly in mouse or human T_H cells, after their formaldehyde fixation/saponin permeabilization. The intracellular staining may be attributed largely to cholesterol-containing membranes of lipid vesicles of varying size (Fig. 4D). Colocalization of ACHA with different intracellular organelle markers was also analyzed and quantified in mouse and human T-cells and macrophages. Intracellularly bound ACHA colocalized with fluorescent brefeldin A [colocalization coefficient (cc), 0.30] (Fig. 4E), a marker of the endoplasmic reticulum and Golgi complex, with the Golgi complex-specific BODIPY-ceramide₅ (cc, 0.32), and more weakly with a marker of lysosomes, LysoTracker (cc, 0.25), but not with markers of mitochondria or cell nuclei (cc, < 0.1) (Fig. 4J).

Cell surface-bound ACHA and the cholesterol-rich lipid raft microdomains

Because cholesterol is known to be enriched in various caveolar or noncaveolar lipid rafts, we intended to investigate how the surface-bound IgG3 ACHAs relate to these membrane microdomains, using snapshot imaging after epitope exposition by papain, as described above. ACHA AC8 strongly colocalized in T-cell membranes with cholera toxin B bound to GM₁ gangliosides (cc, 0.69) (Fig. 4F) and with a protein marker of lipid rafts, Thy1 (cc, 0.55) (Fig. 4G), and, although slightly weaker (cc, 0.39), with transferrin receptors (CD71) marking preferentially the clathrin-coated pit membrane structures (Fig. 4H). In addition, AC8 antibody colocalized substantially with the diIC18 (3) lipid probe (cc, 0.48) as well, reported to preferentially partition into liquid-ordered or gel-phase membrane regions (26) (data not shown), indicating that ACHAs may bind to various cholesterol-rich, ordered microdomains/compartments of the cellular plasma membranes. This is further confirmed by the strong colocalization (cc, 0.63) between AC8 and caveolin-1 in macrophages (Fig. 4I). In contrast, in T-cells, ACHA did not colocalize with CD2 (Fig. 4J), reported as a nonraft protein in unstimulated T-lymphocytes (27).

In addition, AC8 bound to the detergent-resistant membrane fractions isolated from T_H cells and did not bind or bound very weakly to soluble membrane fractions of the same cells, as demonstrated by immunodot blot technique (Fig. 5C). Furthermore, treatment with cholesterol oxidase (COase) resulted in decreased binding of AC8 to both cholesterol and the detergent-resistant fraction on the dot blots. In contrast, no binding was observed to the cold Triton X-100-soluble membrane fraction (Fig. 5C). Deprivation of membrane cholesterol by cholesterol oxidase decreased ACHA binding in both T-helper cells and monocyte-macrophage cells, as assessed by flow cytometry (Fig. 5A, B). Filipin, an antibiotic known to selectively complex membrane cholesterol (23), also slightly decreased AC8 antibody binding to T-cells (Fig. 5A); at the same time, AC8 staining showed the filipin concentration-dependent segregation of cholesterol into large clusters

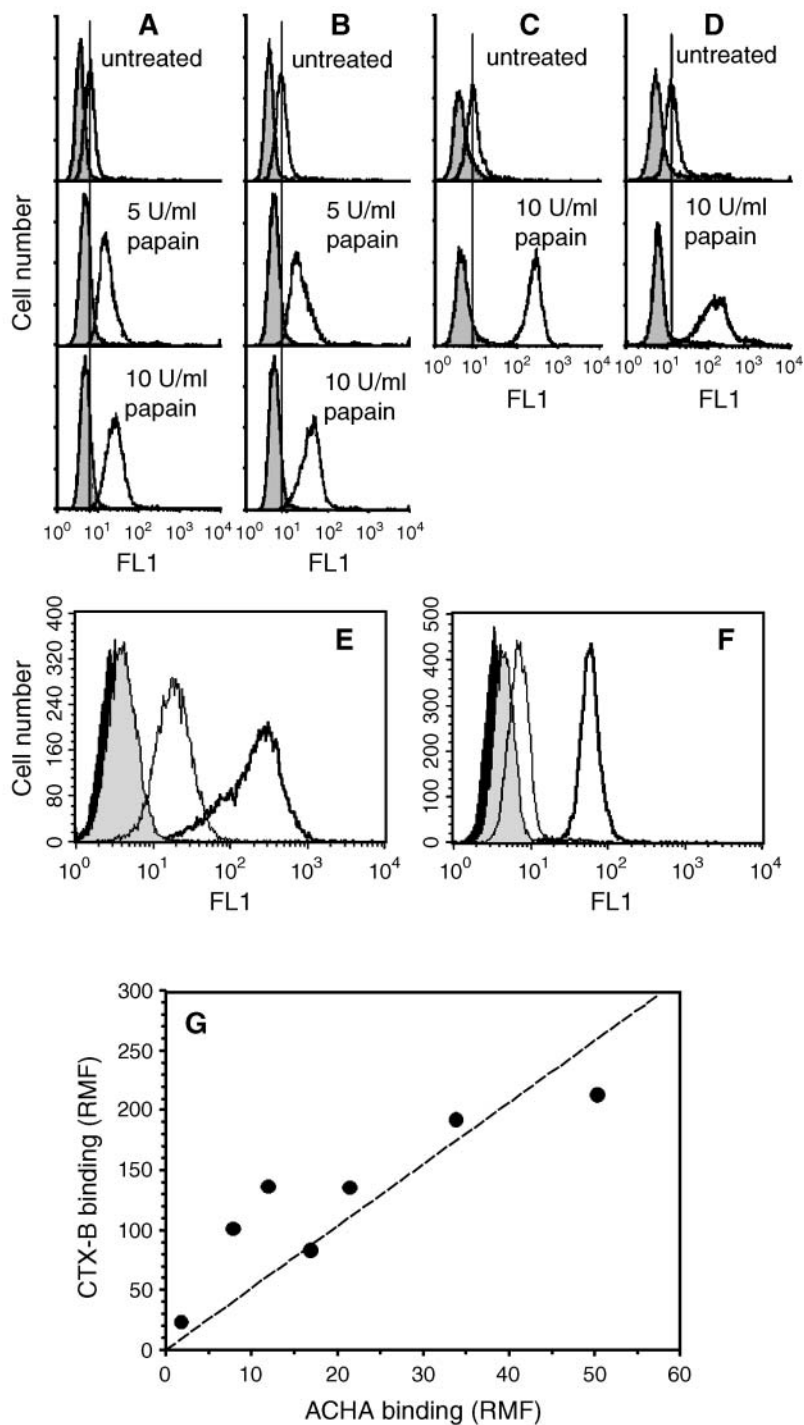


Fig. 3. Flow cytometric analysis of the binding of ACHAs to live murine and human lymphoid and monocyte-macrophage cell lines. A, B: Binding of AC1 (A) and AC8 (B) to murine T-helper cells (2/13), as detected by flow cytometry using Alexa488-conjugated ACHA. Histograms of isotype control (gray areas), ACHA binding to cells without (top) or with 5 U/ml (middle) and 10 U/ml (bottom) papain treatment are displayed. C, D: Binding of AC8 to the MonoMac6 human monocyte-macrophage cell line (C) and the MT-4 (HTLV-1+) human T-lymphoma cell line (D), without or with 10 U/ml papain treatment. Symbols are the same as those used in A and B. E, F: Papain digestion results in a decreased expression of long, protruding proteins, such as CD45R (B220) in B-cells (E) and CD44 in T-cells (F). Isotype controls in the absence (black areas) or presence of papain (gray areas) and immunofluorescence in the absence (thick lines) or presence of papain treatment (thin lines) are shown. G: AC8 binding to various cell types (human and murine B- and T-cells, macrophages) correlates well with GM₁ ganglioside expression of the cells. The correlation between AC8 and CTX binding was strong ($r = 0.89$) among seven different cell types. CTX, cholera toxin B; RMF, relative mean fluorescence.

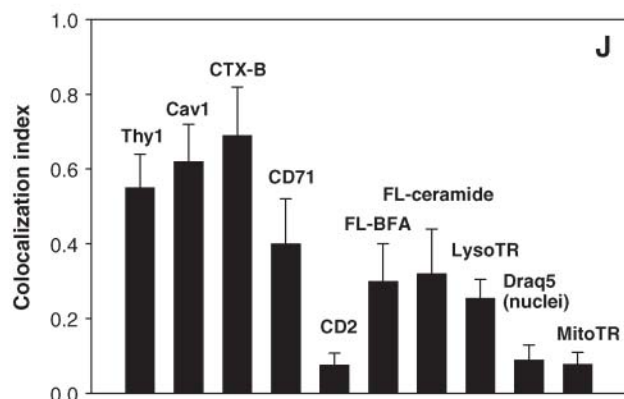
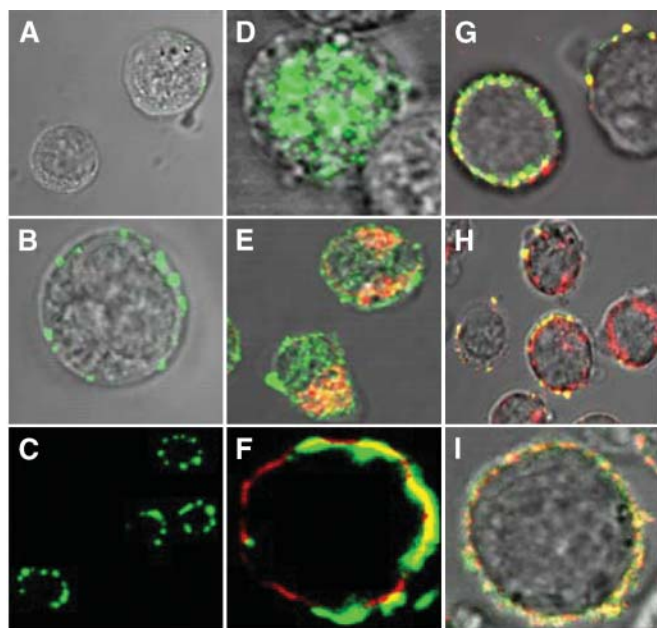


Fig. 4. Binding of AC8 antibody to live cells, by confocal microscopic analysis. Representative images of 2/13 mouse T_H cells labeled with isotype control antibody (A) and Alexa488-AC8 at high (B) or low (C) zoom are shown. The images were acquired using a 60 \times oil-immersion objective (numerical aperture, 1.45). Panels A, B, D, E, G, H, and I are composites of the equatorial fluorescent image slices and the differential interference contrast images of the cells, and panels C and F are fluorescent images. Intracellular staining of saponin-permeabilized 2/13 T_H cells by AC8 is shown in (D). Intracellular bound AC8 showed partial colocalization with a marker of endoplasmic reticulum and the Golgi complex, BODIPY-brefeldin A (E). Representative images show that AC8 colocalizes strongly with CTX-B (GM₁) (F), Thy1 antigen (G), and to a smaller extent with anti-CD71/transferrin receptor (H) in T-cells, as well as strongly with caveolin-1 (I) in macrophages. Colocalization indices of fluorescent AC8 with various fluorescent membrane and intracellular markers are summarized in (J) and expressed as means \pm SD from at least 100 regions of interest. CTX-B, cholera toxin-B.

(Fig. 5D–F). Depletion of membrane cholesterol by methyl- β -cyclodextrin similarly reduced AC8 binding (data not shown).

In quiescent 2/13 mouse T_H cells, the patchy staining by AC8 was uniform (ring-like) along the plasma membrane (Fig. 5D), similar to cholera toxin B staining (Fig. 5G),

with a substantial colocalization of the two labels (Fig. 4J). Activation of the T_H cells with mitogen (concanavalin A) had no effect on the amount of bound cholera toxin B or ACHA (data not shown), although it resulted in a remarkable cell surface redistribution of cholera toxin B, indicating polarization of rafts in the plasma membrane. More than 60% of the T-cells showed highly patchy or cap-like ganglioside/cholera toxin distribution after 24 h of activation (Fig. 5H). AC8 antibody was found highly colocalized with the rafts (cc, >0.85) in activated T-cells (Fig. 5I), indicating that the novel IgG3 ACHAs can monitor the redistribution of cholesterol-rich microdomains in the plasma membranes of intact lymphoid cells upon activation signals.

These data together suggest that the AC8 IgG3-type monoclonal antibody, in addition to its preferential binding to cholesterol-rich raft microdomains at the cell surface, is able to monitor the dynamic redistribution of clustered cholesterol in the plasma membranes of immunocytes during their activation or signal transduction.

DISCUSSION

In this study, we demonstrated that production of not only IgM but also IgG ACHAs can be induced in mice by immunizing them with cholesterol-rich liposomes. In general, IgG3 is primarily produced against antigens with carbohydrate or repeated epitopes. Although the set of 11 generated ACHA clones is statistically insufficient, the fact that we produced exclusively IgM and IgG3 but no other IgG subtypes may indicate that the immunogenicity of cholesterol is somehow related to these classes of epitopes. This is supported by the structural similarities between the 3 β -hydroxyl group and its environment in cholesterol and some surface-associated carbohydrate structures of pathogens, as well as by the periodically repeated (clustered) arrangement of cholesterol.

An earlier monoclonal IgM-type ACHA (monoclonal antibody 2C5-6) showed selectivity in its binding to aggregated cholesterol but not to strictly defined and organized surfaces of cholesterol monohydrate crystals. In contrast, other monoclonal IgMs selected against cholesterol crystals, recognizing a given pattern of epitopes present on the crystal faces. A cholesterol concentration of 10 μ g/ml was reported as a critical threshold for obtaining a significant amount of microcrystals (4). Although no direct binding studies have been performed on crystalline cholesterol, the affinity of our two novel IgG ACHAs did not significantly depend on the cholesterol concentration being in the range of 0.1–10 μ g/ml. This suggests that the IgG-type ACHAs AC8 and AC1 are less sensitive to the aggregation state of cholesterol than the previously developed IgM isotype ACHAs.

Dijkstra and coworkers (19) reported that cholesterol-immunoreactive sera and a monoclonal anti-cholesterol IgM (2C5-6) were both specific to cholesterol and structurally similar sterols containing a 3 β -hydroxyl group. In accordance with this observation, we found that the anti-

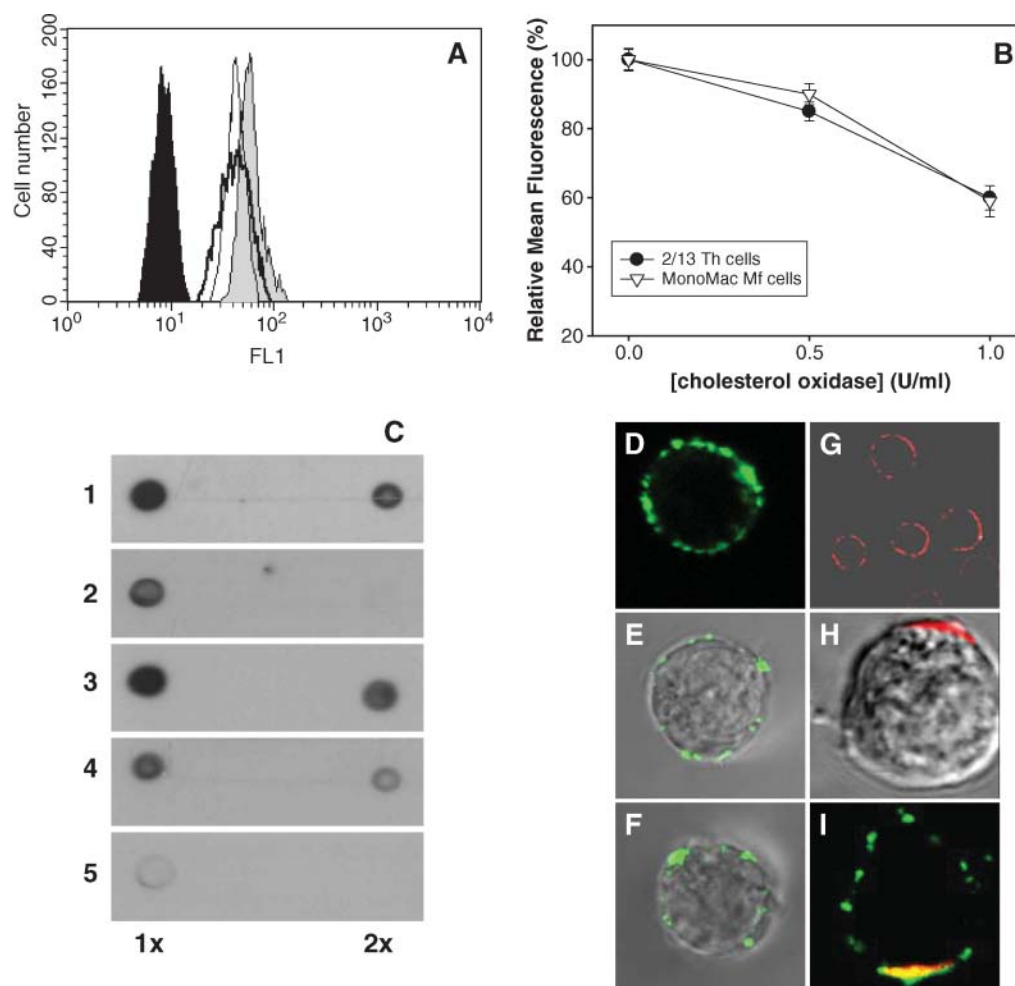


Fig. 5. AC8 binding to T-cells and macrophage cells is sensitive to membrane cholesterol/raft modulations. **A:** Filipin (10 $\mu\text{g/ml}$; thick line) or cholesterol oxidase (1 U/ml; thin line) reduces AC8 binding to the surface of papain-treated 2/13 T_H cells. Immunofluorescence of isotype control (black area) or AC8-labeled (gray area), papain-treated cells is also shown. **B:** Cholesterol oxidase dose-dependently reduces AC8 binding to papain-treated 2/13 T_H lymphocytes (closed diamonds) and MonoMac6 (open inverted triangles) human monocyte-macrophage cells. **C:** Immunodot blot analysis of detergent-resistant (gels 3, 4) and detergent-soluble (gel 5) fractions isolated from T_H cells and cholesterol (gels 1, 2) as a control. Samples were untreated (gels 1, 3, 5) or treated with cholesterol oxidase (gels 2, 4) and dropped onto the nitrocellulose membrane in small (2 μl) amounts either directly or at 2 \times dilution. **D–I:** Treatment of T-cells with 5 $\mu\text{g/ml}$ (E) and 10 $\mu\text{g/ml}$ (F) filipin results in a gradual segregation of membrane cholesterol relative to untreated control cells (D). 2/13 T_H cells are stained in a uniformly ring-like, patchy manner with cholera toxin B (G). Activation of T_H cells with concanavalin A mitogen results in capping of cholera toxin B, as shown by the representative image in (H). AC8 is strongly copolarized with cholera toxin B in the membrane of mitogen-activated T-cells (I). Error bars represent SEM.

cholesterol IgG monoclonal antibodies bound efficiently only to those cholesterol-like sterols that contain a 3β -hydroxyl group. However, additional chemical moieties or the polarity of the sterols may also influence antibody binding, consistent with the lack of binding to 25-hydroxycholesterol bearing the free 3β -hydroxyl moiety. The two monoclonal ACHAs (AC8 and AC1) differed slightly in their binding preference to the tested sterols, suggesting that, in spite of the small epitope size, two antibodies with different fine specificities could be generated.

Our monoclonal IgG ACHAs bound to human lipoproteins HDL, LDL, and VLDL alike. In contrast to previous studies with IgM, no strict selectivity was found in the

binding to LDL and VLDL over HDL. Dijkstra et al. (19) reached their conclusion about binding selectivity on the basis of bound antibody per total (or free) cholesterol. However, the total cholesterol content (free plus esterified cholesterol) per particle mass, the particle size, the ratio of surface free cholesterol to total cholesterol, and the protein-to-lipid ratio may all vary strongly among the different lipoproteins. Moreover, these factors together may influence the available surface area for antibody binding. When the binding of our IgG ACHAs to the different lipoproteins was normalized for equivalent surface area, we obtained similar values, and the avidity to HDL was only slightly lower than that to LDL or VLDL. This is consistent

with the higher surface density of proteins in HDL, because the proteins may shield part of the surface cholesterol epitopes, and is also indicative of an important difference between IgM- and IgG-type ACHAs.

High serum levels of low density lipoproteins and low serum concentrations of HDL are independent risk factors for atherosclerotic diseases (28–32). The previously reported monoclonal ACHA binding to LDL and VLDL but not to HDL (19) led to the hypothesis (33) that naturally occurring ACHAs may have a role in the selective clearance of LDLs (“bad cholesterol”). That the IgG ACHAs generated in this work also bind to HDL, however, suggests that the role of natural ACHAs in the pathogenesis of atherosclerosis cannot be simplified. This is further supported by the clinical findings of high ACHA serum levels in patients with coronary heart disease (9) but low ACHA levels in patients with cerebrovascular diseases (29, 31) compared with healthy individuals.

As cholesterol is a fundamental component of cell membranes, it is an intriguing question whether natural ACHAs can bind to cell surfaces under physiological or pathological conditions. Previously, monoclonal IgM ACHAs were reported to react with plasma membrane cholesterol microdomains of macrophages responsive to changes in cholesterol trafficking. These IgMs bound to the cells only after artificial cholesterol enrichment by various treatments, suggesting that they define cholesterol arrays that are presumably not identical to lipid rafts (5, 24).

The novel IgG monoclonal antibodies AC1 and AC8 bound spontaneously, although weakly, to intact human and murine lymphoid or monocyte-macrophage cells. A limited papain digestion, removing some long extracellular protein domains from the cell surface, strongly enhanced their avidity, indicating that the IgG ACHAs bind to “extremely small epitopes” that are mostly shielded by the long, protruding membrane proteins. The higher level of AC8 binding to monocyte-macrophage cells versus lymphoid cells is likely attributable to the easier access to cholesterol epitopes in “opened” caveolar structures compared with the crowded surface of lymphocytes.

Exposure of cholesterol epitopes, however, may also change during apoptosis and the budding of enveloped viruses or under pathological conditions (e.g., tumors), because all of these processes may alter molecular composition, curvature, or the “surface smoothness” of the involved membrane regions. Recognition of the exposed cholesterol epitopes by naturally occurring cholesterol-specific antibodies may have further physiological consequences, such as activation of the complement and/or phagocytic system to eliminate dead cells as well as virus particles. Such altered cholesterol exposure may also be relevant in the increased level of ACHAs observed under pathological conditions (8–10, 34).

The high degree of colocalization between the IgG ACHA and cholera toxin B, anti-Thy1, or anti-caveolin-1 antibodies indicates that rafts or caveolae can be considered preferential binding sites for ACHAs in the plasma membrane. Although in lymphocytes and macro-

phages cholesterol can be found in fluid-phase membrane regions as well, it is locally enriched (clustered) in the noncaveolar and caveolar rafts or, to a lesser extent, in other functional membrane domains, such as chla-thrin-coated pit structures (6), consistent with our findings. Aggregation of lipid rafts by T-cell activation signals has been described (6). Thus, the strongly patchy nature of membrane staining by ACHA and its copolarization (capping) with the lipid raft marker cholera toxin B upon T-cell activation, together with a substantial degree of colocalization between ACHA and other lipid raft markers, all suggest that AC8 antibody is capable of marking lipid raft microdomains and even monitoring their activation-induced redistribution in the plasma membranes of live lymphoid cells.

In contrast to the IgM antibodies reported by Kruth et al. (5), our data show that the IgG ACHAs bind to T- or B-cell membrane microdomains displaying detergent resistance and enriched in known markers of lipid rafts. These properties of the new IgG ACHAs allow further possible applications in the study of lipid raft functions.

In addition, the structural integrity of raft/caveola microdomains, stabilized by cholesterol, is essential in various immunological functions, such as antigen presentation (35–37), T-cell receptor-mediated signal transduction (36), and the uptake of viral (e.g., human immunodeficiency virus) and other pathogens or particles by phagocytes (16, 37). This raises the question of whether binding of ACHAs to the membrane microdomains of these cells can modulate these processes and, if so, how. Our preliminary results obtained with an antigen-presenting cell (APC)-T_H cell immunological synapse model (21), showing AC8-augmented antigen presentation and an enhanced phagocytosis of opsonized yeast cells by macrophages upon AC8 antibody binding, suggest that AC8 may have potential to modulate various immune functions dependent somehow on cholesterol-rich lipid rafts. Such modulatory effects are limited to those immunocytes to which ACHAs can bind spontaneously to a sufficient extent (e.g., APCs, macrophages). Indeed, no modulatory effect of ACHA on T-cell activation was observed in the APC-T_H cell immunological synapse if the T_H cells were preincubated with ACHAs instead of the APCs (38).

In conclusion, we generated two IgG3 isotype clones of ACHAs, AC1 and AC8, that were characterized in detail in both cell-free and cellular systems and have shown their potential to mark and monitor changes in the cell surface distribution of cholesterol-rich caveolar or noncaveolar lipid rafts. Consistent with their substantially enhanced binding to the surface of various immunocytes upon moderate papain digestion, we propose that IgG ACHAs, in contrast to IgM isotypes, may function by directly modulating several lipid raft-dependent immune functions, especially under specific physiological or pathological conditions, altering the epitope accessibility of their target cells. Further detailed investigations are required, however, to validate this and other possible functions of ACHAs in modulating the immune response; these are currently running in our laboratories. ■

The authors gratefully acknowledge Árpád Mikessy for his contribution to the immunization experiments and Dr. Zoltán Prohaszka for valuable discussions. This work was supported by Hungarian National Science Fund Grants TS044711 (J.M., G.L.), T043613 (G.L.), T049696 (J.M.), and F049164 (L.C.) and by Grant ETT 195/2003 from the Hungarian Ministry of Health (L.R., G.F.).

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