

Characterization of Recombinant Monoclonal IgA Anti-PDC-E2 Autoantibodies Derived From Patients With PBC

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Primary biliary cirrhosis (PBC) is an autoimmune liver disease characterized by the presence of autoantibodies to mitochondria (AMA). Recent evidence suggests that PBC develops after a locally driven response in the mucosa, where immunoglobulin A (IgA) is the dominant antibody isotype. In this study, we produced recombinant pyruvate dehydrogenase complex (PDC-E2)-specific dimeric human IgA monoclonal antibodies (mAbs) in a baculovirus expression system. By using 2 anti-PDC-E2 IgG mAbs derived from patients with PBC, we constructed 2 recombinant baculoviruses, each containing heavy chains with the C α constant region. These were simultaneously co-infected into Sf9 insect cells with recombinant baculovirus containing the J chain. A sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) immunoblotting profile of the IgA using a 6% nonreducing gel verified the dimeric nature of the autoantibodies. Both recombinants retained their original specificity for PDC-E2. In addition, the antibody showed a mitochondrial staining pattern in HEp2 cells and apically stained the biliary epithelial cells (BECs) in the liver of a patient with PBC but not a normal patient. Transcytosis experiments performed using human polymeric immunoglobulin receptor (pIgR) expressing Madine-Darby canine kidney (MDCK) cells showed that one of the recombinants showed a high degree of colocalization with PDC-E2. In conclusion, these data provide further support of the hypothesis that PDC-E2-specific IgA may enter biliary epithelial cells of PBC patients via the pIgR and complex with PDC-E2, thereby potentially contributing to the pathology of BECs. Moreover, this recombinant PDC-E2-specific mAb provides a tool for further determination of the role of anti-PDC-E2 IgA in the pathogenesis of PBC. (HEPATOLOGY 2002;36:1383-1392.)

Primarily biliary cirrhosis (PBC) is a chronic autoimmune liver disease characterized by the presence of high-titer antimitochondrial antibodies (AMA) and the progressive inflammatory destruction of intrahe-

patic bile ducts.^{1,2} The autoantigens recognized by AMA have been identified as members of the 2-oxo acid dehydrogenase enzyme family located in the inner mitochondrial membrane.³⁻⁵ The dihydrolipoamide acetyltransferase (E2 component) of pyruvate dehydrogenase complex (PDC) is the most common autoantigen and reacts with 95% of PBC sera.

One critical question in the pathogenesis of PBC is why the biliary epithelial cells are targeted despite the ubiquitous presence of PDC. Several reports have suggested that PBC is a mucosal disease with generalized epithelitis. In fact, immunoglobulin (Ig)A AMA are readily detected in the bile, saliva, and urine from patients with PBC.⁶⁻⁸ It also has been suggested that anti-PDC-E2 IgA may be involved in the destruction of bile duct epithelial cells in PBC either by binding PDC-E2 and forming immune complexes or by trafficking to mitochondria during the normal course of transcytosis.⁹ The process of transcytosis, which is used by the body to deliver protective immunoglobulins to its mucosal surfaces, involves the movement of polymeric IgA and IgM through the cytoplasm from the basolateral to the apical domain and is mediated by the polymeric immunoglob-

Abbreviations: PBC, primary biliary cirrhosis; AMA, antimitochondrial antibodies; PDC-E2, pyruvate dehydrogenase complex; Ig, immunoglobulin; pIgR, polymeric immunoglobulin receptor; MDCK, Madine-Darby canine kidney; PCR, polymerase chain reaction; V_H, variable heavy-chain; SDS, sodium dodecyl sulfate; Sf9, Spodoptera frugiperda; Ars, anti-p-azophenylarsonate; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; TBS, Tween-buffered saline; mAb, monoclonal antibody; BEC, biliary epithelial cells.

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ulin receptor (pIgR) that is only expressed on epithelial cells. The pIgR is distinct from other Fc receptors in that it does not promote a proinflammatory response when interacting with ligand; its main function is one of translocation not activation.

One limitation in studying the potential ability of IgA AMA to gain entry via transcytosis in epithelial cells has been the lack of a monospecific poly IgA preparation because efficient transcytosis requires polymers of IgA containing the J chain. Unfortunately, although serum is the most readily available source of IgA, it is poor in polymeric IgA content. Furthermore, it is very difficult, if not impossible, to make affinity-purified IgA. Therefore, we produced 2 dimeric human IgA monoclonal anti-PDC-E2 antibodies by using a baculovirus expression vector system, and examined the specificity and pattern of reactivity of this recombinant IgA as well as its colocalization with antigen in a pIgR-expressing Madine-Darby canine kidney (MDCK) cell line after transcytosis.

Materials and Methods

Synthesis of the IgA Coding Region and Construction of a Baculovirus Transfer Vector. Recombinant human IgA antibodies were constructed by using 2 sources for genes encoding the variable regions of PDC-E2-specific IgA, LC5, and PD2. We previously reported the generation of LC5, a human IgG combinatorial antibody with specificity for PDC-E2 derived from the lymph node of a patient with PBC¹⁰ by using polymerase chain reaction (PCR) and selective synthesis of the recombinant Fab. PD2, also a PBC patient-derived antibody, was made originally by immortalization of an isolated plasma cell.¹¹ Variable heavy-chain (V_H) and variable light-chain regions for PD2 were amplified from the plasmid PD2/PD2, which contains the heavy- and light-chain sequences of this antibody. Hereafter, we will describe the procedures in reference to LC5 for simplicity because, with the exception of the initial variable region amplification, the general procedures for LC5 and PD2 were identical and are shown in Fig. 1.

DNA fragments corresponding to the V_H region of LC5 were amplified by PCR from *pH360-ExLC5heavy* and DNA fragments corresponding to $C\alpha 1$ were amplified by PCR from *pH360-Exheavy*. By using the PCR overlap extension method,¹² the V_H region and $C\alpha 1$ were joined so that the acquired nucleotide sequence consisted of signal sequence, V_H region, and $C\alpha 1$. This product was then cloned into the pFASTDUAL vector (Gibco BRL, Rockville, MD). The DNA fragment encoding the light chain (V region and $C\lambda$ region) of LC5 was amplified from *pH360-ExLC5light* so that the PCR product con-

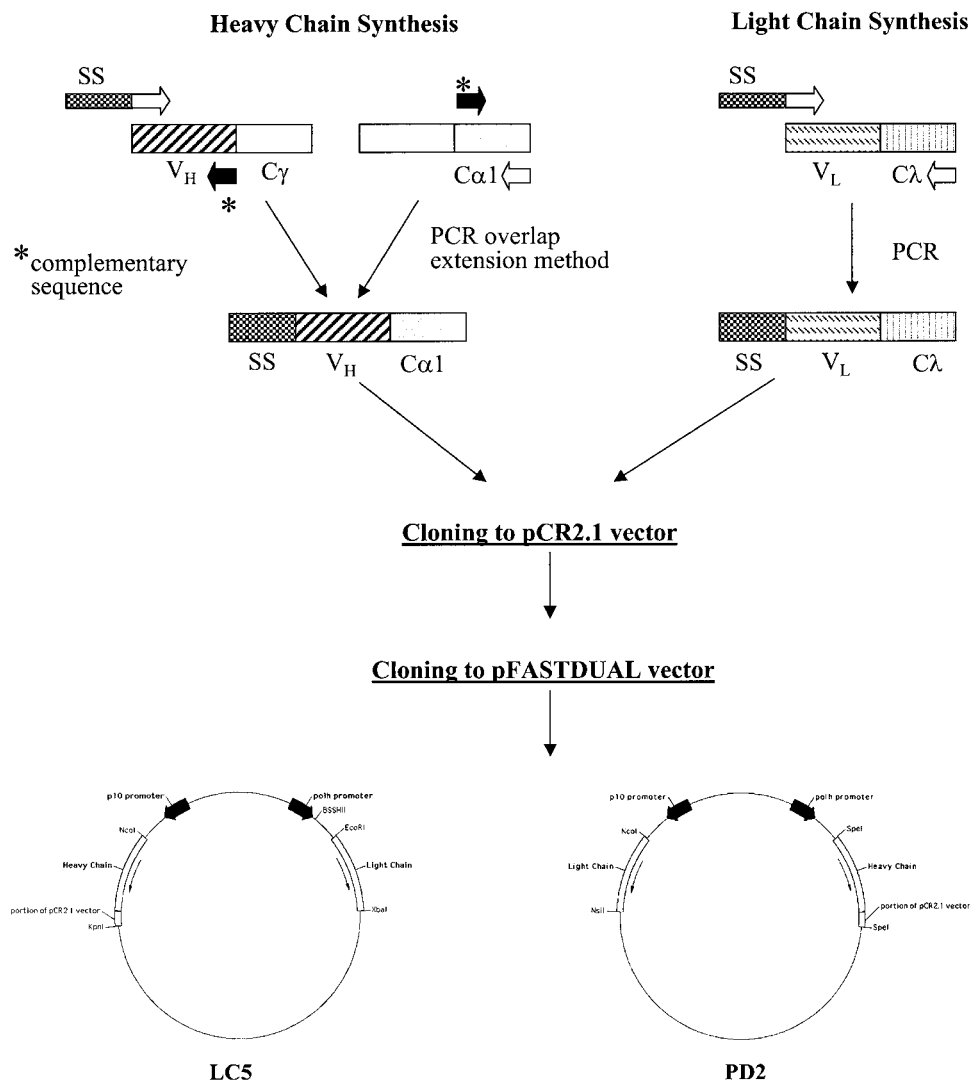
sisted of signal sequence, V region, and $C\lambda$ region. The light chain was cloned into the pFASTDUAL vector. Then both the vectors containing the heavy-chain sequence of LC5 and the light-chain sequence of LC5 were combined into one pFASTDUAL vector and cloned. The acquired vector contained both the heavy- and light-chain sequence of LC5 under their respective promoters.

Production of Recombinant Viruses. Competent DH10Bac *Escherichia coli* cells (Gibco BRL) were transformed with recombinant pFASTDUAL by heat shock. Approximately 1 ng of recombinant donor plasmid (in 5 μ L) was added and gently mixed with 100 μ L of thawed DH10Bac cells. The mixture was incubated on ice for 30 minutes and subjected to heat shock in a 42°C water bath for 45 seconds, then chilled on ice for 2 minutes. Then 900 μ L SOC medium was added and the mixture was shaken in an incubator at 37°C at 225 rpm for 4 hours. One hundred microliters of each mixture was spread on L-Broth-agar plates containing 100 μ g/mL Blue-gal, 40 μ g/mL isopropylthiogalactoside, 50 μ g/mL kanamycin, and 10 μ g/mL tetracycline. Plates were incubated for 24 to 48 hours at 37°C. White colonies were picked up and inoculated into 2 mL L-Broth medium with 50 μ g/mL kanamycin, 7 μ g/mL gentamycin, and 10 μ g/mL tetracycline.

The procedures for purification of the bacmid containing the immunoglobulin gene, transfection, titration, and amplification of virus were performed according to the manufacturer's directions. Purification was performed by using the alkali-sodium dodecyl sulfate (SDS) method. DNA was extracted by isopropanol and finally dissolved in 40 μ L Tris-EDTA. The existence of bacmid-containing inserts of the appropriate size was confirmed by PCR according to the BAC-TO-BAC Baculovirus Expression Systems method (Gibco BRL).

Spodoptera frugiperda (Sf9) cells (9×10^5 Sf9 cells in a 35-mm culture dish) were transfected with 5 μ L of bacmid and incubated for 4 to 5 hours at 27°C before changing into bacmid free media containing 2.5% fetal bovine serum and 150 μ g/mL kanamycin. Three to 4 days later, the medium was collected, centrifuged at 2000g for 5 minutes, and the supernatant harvested. The viral titer in the supernatant was determined by a plaque assay as described previously.¹³ For virus amplification, virus was added to cells plated at 100% confluency. After 3 to 4 days the medium was collected, centrifuged at 2000g for 5 minutes, and the supernatant collected. The recombinant baculovirus stock, which contains the human J chain gene, was kindly provided by Dr. J. Donald Capra. Titration and amplification of the virus containing the gene for human J chain were performed as mentioned earlier.

Fig. 1. Recombinant IgA, LC5, and PD2 were generated by first amplifying the v-regions from the parent vectors. To switch the isotype of the antibodies, C α 1 was amplified by using a 5' primer containing a sequence complementary to the 3' portion of the desired variable region, whereas the 3' primer of the V_H region contained a sequence complementary to the 5' portion of C α 1. The signal sequence (ss) was added to both the heavy and light chain by incorporating it into the 5' primer sequence (restriction sites also were added to primers to facilitate molecular manipulation). Subjecting the newly amplified V_H region and C α 1 to the PCR overlap extension method resulted in the generation of a recombinant IgA heavy chain expressing the desired variable region. After cloning the heavy and light chains into separate pCR2.1 vectors, immunoglobulin genes and pFASTDUAL vectors were digested by the appropriate restriction enzymes, gel purified, and ligated together. This resulted in 2 pFASTDUAL vectors for each antibody, one containing the heavy chain and the other the light chain. To improve transfection efficiency, the heavy and light chain genes were combined onto one pFASTDUAL vector by digesting, gel purifying, and ligating the 2 portions of the vectors together.



The Sf9¹⁴ cells were maintained in ExCell 401 medium (JRH Biosciences, Lenexa, KS) supplemented with 2.5% fetal bovine serum at 27°C as described,¹³ by using 1.6×10^5 cells/cm² for plated cultures, and 2.5×10^6 cells/mL for spinner flask cultures (set at 100 rpm). These insect cells then were co-infected simultaneously with a recombinant baculovirus containing recombinant immunoglobulin (either LC5 or PD2) and the virus containing J chain by using more than 10 multiplicity of infection for each virus. To generate a control antibody, viruses that produced the heavy chain and light chain of anti-*p*-azophenylarsonate (Ars) IgA (a gift from Dr. J. Donald Capra) also were used to infect Sf9 cells. Amplification and co-infection of these 2 viruses were similar to that of LC5 described earlier.

Harvest and Purification of Supernatant. A sandwich enzyme-linked immunosorbent assay (ELISA), using anti-human IgA Ab as the capture agent, was used to determine the time-course of IgA production after infec-

tion. The optimal concentration of LC5 and PD2 IgA in the medium was acquired at 90 hours (data not shown). Therefore, all subsequent supernatant fluids from the culture of LC5 and PD2 IgA were collected 90 hours after infection. Harvested medium was centrifuged at 2000g for 5 minutes, and supernatant applied to a .05- μ m hollow fiber filter (Spectrum, Rancho Dominguez, CA) to eliminate virus. The collected medium was then applied to a 50-kd hollow fiber filter (Spectrum) and proteins greater than 50 kd were collected. Finally, for biotinylation and subsequent use in nonreducing SDS/6% PAGE, the concentrated recombinant IgA was applied to a jacalin column (Pierce, Rockford, IL) and dialyzed against phosphate-buffered saline. The medium containing the control Ars was collected 72 hours after infection, and concentrated by both a .05- μ m and 50-kd hollow fiber filter as described for LC5 IgA.

ELISA. The reactivity of our recombinant IgA, serum IgA, and, for purposes of control, commercial IgA

(Pierce) against the recombinant mitochondrial autoantigens PDC-E2, OGDC-E2, BCOADC-E2, and E3BP were studied by using methods described previously.^{15,16} We used a peroxidase-conjugated goat anti-human IgA secondary antibody (Biosource, Camarillo, CA) at a pre-determined optimal dilution of 1:10,000.

The recombinant PDC-E2 spans amino acids 91 to 228 of PDC; BCKD-E2, from 1 to 118; OGDC-E2, from 1 to 147; and E3BP, from 6 to 84. In addition, we used a full-length recombinant PDC-E2 for some experiments.

Immunoblotting. Purified beef heart mitochondria, recombinant peptides, bacterial lysates, and purified recombinant IgA LC5 and PD2 were analyzed by SDS-PAGE. For immunoblotting, proteins were transferred onto a .45- μm NitroPure membrane (Micron Separations Inc., Westborough, MA) in 20% methanol/25 mmol/L Tris/191.8 mmol/L glycine. Membranes were blocked with 3% dehydrated skim milk in phosphate-buffered saline. Recombinant IgA LC5 and PD2 were assayed by nonreducing SDS-PAGE using 6% gels, and detected by using goat anti-human IgA conjugated to horseradish peroxidase (Biosource) at a 1:3,000 dilution, followed by chemiluminescence using Supersignal Substrate Kit (Pierce). The ratio of monomeric to dimeric IgA was determined by densitometric analysis. The specificity of LC5 and PD2 was determined by using recombinant mitochondrial antigens and beef heart mitochondria. The antigens were separated on a reducing 10% SDS-PAGE gel, blotted, and incubated with concentrated recombinant IgA. After washing with phosphate-buffered saline/0.05% Tween, beef heart mitochondria and recombinant PDC-E2 blots were incubated with mouse anti-human IgA conjugated to horseradish peroxidase (Zymed, S. San Francisco, CA) at a 1:1,000 dilution. Recombinant OGDC-E2, recombinant BCOADC-E2, and recombinant E3BP were incubated with goat anti-human IgA conjugated to horseradish peroxidase (Biosource) at a 1:20,000 dilution. All samples were visualized by chemiluminescence as described earlier.

HEp2 Staining. After blocking with avidin-biotin block (Vector Laboratories, Inc., Burlingame, CA), medium containing recombinant dimeric IgA LC5 with J chain and, for purposes of control, medium containing anti-Ars IgA or IgA purified from PBC sera were added to HEp2 cells (ANA test system; Immuno Concepts, Sacramento, CA). The 2 recombinant samples were applied undiluted, and IgA purified from PBC was applied at 50 $\mu\text{g}/\text{mL}$. After incubation at 37°C and washing, mouse anti-human IgA-biotin (Pharmingen, San Diego, CA) was added at a 1:100 dilution. The slides were washed and avidin-Alexa 488 (Molecular Probes Inc., Eugene, OR)

was added at a 1:100 dilution and incubated at 37°C, followed by washing and mounting with Fluoroguard anti-fade reagent (BioRad, Hercules, CA).

Immunohistochemical Staining of Human Liver. After collection, liver tissue was fixed in 10% neutral buffered formalin and paraffin embedded; 10- μm sections were cut and mounted on microscope slides. The sections were deparaffinized by heating to 65°C for 1 hour, and followed by successive treatments with xylene (100%) and ethanol (100%, 90%, 70%). The sections were then washed for 5 minutes in Tris-buffered saline (TBS), and nonspecific binding blocked by using diluted horse serum. Biotin-labeled LC5, diluted 1:5 in TBS, was added to sections and incubated overnight at room temperature in a humid chamber. The sections were then washed 2 times for 5 minutes in TBS. Last, the sections were incubated in alkaline phosphatase-conjugated streptavidin-biotin complex (Vector Laboratories) for 30 minutes. The slides were then washed 2 times in TBS for 5 minutes, and Vector Red Substrate (Vector Laboratories) was added for 20 minutes. Sections were washed in deionized water for 5 minutes, and then cover slips were applied by using a 1:1 solution of glycerol:water. The slides were viewed by confocal microscopy with a Bio-Rad MRC 1024 ES laser confocal microscope (Bio-Rad).

Colocalization of Recombinant IgA and PDC-E2 in Epithelial Cells. MDCK cells expressing the pIgR were plated on 12-mm Transwell filters (Corning Inc., Corning, NY) and grown to confluency. Purified dimeric myeloma IgA₁ (The Binding Site, San Diego, CA) and PD2 IgA at a concentration of 5 $\mu\text{g}/\text{mL}$, as well as serum IgA at a concentration of 50 $\mu\text{g}/\text{mL}$, were added to the basolateral side of the cells and incubated for 15 minutes at 37°C. Staining was performed as described previously⁹ with bovine serum albumin used as the protein in the blocking solution. For IgA staining, goat anti-human IgA (Zymed) was used followed by donkey anti-goat Alexa 488 conjugate (Molecular Probes). For PDC-E2 staining, monoclonal antibody (mAb) C355.1 (PDC-E2 specific) cell culture supernatant was used followed by Alexa-594 conjugated goat anti-mouse IgG F(ab')₂ (Molecular Probes). Alexa-488 and -594 fluorochromes were chosen based on their well-separated emission spectra to avoid false-positive colocalization. Samples were mounted onto glass slides with Fluoroguard antifade reagent (Bio-Rad) under glass coverslips. Then the dual-stained cells were visualized on a Bio-Rad Radiance 2100 laser confocal microscope (Bio-Rad). The green channel and red channel z-series sections (0.5 μm) were collected simultaneously and colocalization was determined in 10 IgA-positive cells per sample by using Laserpix v.4.0 software (Bio-Rad).

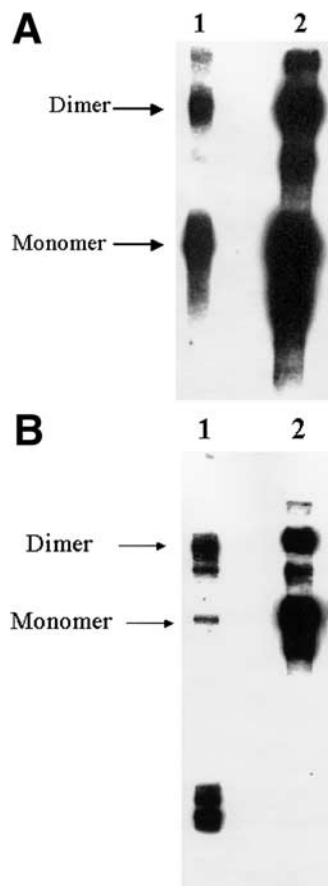


Fig. 2. Nonreducing 6% SDS-PAGE and anti-IgA immunoblot analysis. **Lane 1** contains recombinant IgA LC5 purified by (A) jacalin column and (B) PD2 enriched by ammonium sulfate precipitation. (A and B) **Lane 2** contains IgA purified from a PBC patient. Dimeric and monomeric IgA can be seen in both the LC5 and PD2 preparations.

Results

Synthesis, Assembly, and Secretion of Dimeric PDC-E2 IgA. The baculovirus transfer vector pFAST-DUAL has 2 promoters and 2 multiple cloning sites. This allows the simultaneous expression of both heavy and

light chains of a particular immunoglobulin from a single recombinant virus. As expected, both the recombinant viruses expressing the heavy and light chains of LC5 or PD2 replicated normally ($>3 \times 10^8$ pfu/mL), indicating that recombinant protein expression was not toxic for the insect cells. By both immunoblot analysis and ELISA, the recombinant viruses were shown to induce expression of monomeric IgA antibodies with specificity for PDC-E2 (data not shown). An immunoblot of a nonreducing 6% SDS-PAGE (Fig. 2) revealed that the co-infected insect cells produce and secrete both dimeric and monomeric IgA. The ratio of the synthesis of dimeric to monomeric forms of IgA, determined by densitometric analysis of Western blots, was higher for PD2 (5:1) than that of LC5 IgA (1:4). No detectable multimeric forms of IgA were produced when insect cells were infected with virus containing the immunoglobulin of LC5 or PD2 without J chain (data not shown).

Specificity of Recombinant IgA. As shown in Table 1, recombinant LC5 IgA has strong reactivity against PDC-E2 and weak reactivity against E3BP, but did not react against OGDC-E2 and BCOADC-E2 by ELISA. LC5 IgA reacted by blot to both recombinant BCOADC-E2 and OGDC-E2 but not by ELISA; this likely is because proteins in an ELISA retain their native conformation as compared with Western blots, which strongly denature antigen. Although this recombinant IgA reacted against the inner lipoic domain of PDC-E2 (optical density [OD], 0.453 ± 0.012), it failed to show detectable reactivity against the outer lipoic domain (OD, 0.001 ± 0.001), the E1/E3 binding site (OD, -0.001 ± 0.000), and the inner core and catalytic site (OD, 0.005 ± 0.002). PD2 IgA recognized PDC-E2 (data not shown) but the fine-specificity bound by this recombinant was not mapped. We have shown previously that PD2 recognizes both the inner lipoic domain (in a lipoic acid-dependent manner), and the outer lipoic domain of PDC-

Table 1. Specificity of Recombinant LC5 IgA

Antibody	Recombinant Mitochondrial Autoantigen				Control Antigen Metel*
	PDC-E2	OGDC-E2	BCKD-E2	E3BP-E2	
LC5 IgA	$1.190 \pm 0.016^\dagger$	-0.001 ± 0.001	0.003 ± 0.001	0.102 ± 0.003	0.001 ± 0.002
Anti-Ars IgA	0.003 ± 0.001	0.002 ± 0.001	0.002 ± 0.001	-0.001 ± 0.001	0.001 ± 0.001
PBC serum No. 9	0.109 ± 0.001	0.049 ± 0.001	0.049 ± 0.001	0.067 ± 0.001	0.043 ± 0.001
PBC serum No. 10	0.678 ± 0.001	0.140 ± 0.002	0.655 ± 0.008	0.212 ± 0.009	0.064 ± 0.000
PBC serum No. 12	0.174 ± 0.001	0.110 ± 0.005	0.373 ± 0.003	0.111 ± 0.003	0.044 ± 0.000
PBC serum No. 14	0.596 ± 0.008	0.143 ± 0.003	0.163 ± 0.002	0.142 ± 0.000	0.083 ± 0.004
PBC serum No. 17	0.887 ± 0.017	0.128 ± 0.001	0.150 ± 0.005	0.160 ± 0.001	0.083 ± 0.006
PBC serum No. 27	0.259 ± 0.002	0.016 ± 0.001	0.030 ± 0.002	0.017 ± 0.001	0.018 ± 0.007
Pierce IgA (control)	0.011 ± 0.001	0.014 ± 0.003	0.015 ± 0.002	0.015 ± 0.002	0.017 ± 0.003

*Control antigen derived from a recombinant shrimp tropomyosin.

† OD value at 405 nm (mean \pm SEM).

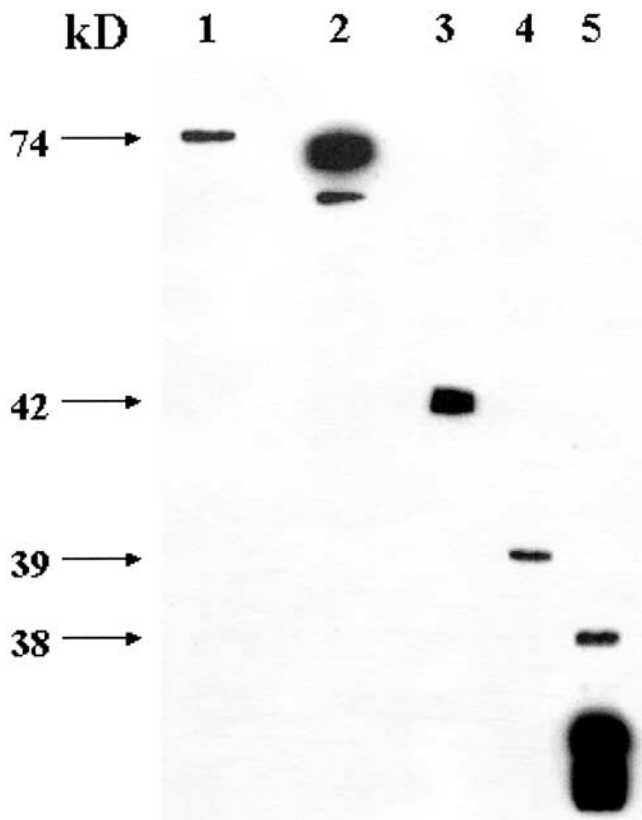


Fig. 3. Reducing 10% SDS-PAGE and recombinant IgA LC5 immunoblotting against beef heart mitochondria and recombinant autoantigens. **Lane 1**, beef heart mitochondria was probed with LC5 and gave a band at 74 kd that corresponds to PDC-E2. **Lane 2**, full-length recombinant PDC-E2, as well as an abbreviated form due to an internal Shine-Dalgarno sequence in the messenger RNA, was recognized by LC5. **Lane 3**, LC5 recognized recombinant OGDC-E2 at 42 kd. **Lane 4**, LC5 recognized recombinant BCOADC-E2 at 39 kd. **Lane 5**, LC5 also recognized recombinant E3BP at 38 kd.

E2.¹⁷ Although recombinant anti-Ars IgA was shown to have reactivity against Ars as expected (data not shown), it did not have reactivity against any of the mitochondrial recombinant peptides.

By immunoblot analysis, both the recombinant IgA LC5 and PD2 reacted against native PDC-E2 in beef heart mitochondria. Immunoblotting against recombinant autoantigens showed that recombinant IgA LC5 had reactivity against PDC-E2, OGDC-E2, BCOADC-E2, and E3BP (Fig. 3). The reactivity of recombinant PD2 against beef heart mitochondria is shown in Fig. 4. Immunoblotting against bacterial lysates showed that recombinant IgA LC5 does not have detectable reactivity against *E. coli*, *Neisseria*, and *Lactobacillus* (data not shown), suggesting that IgA LC5 does not cross-react against PDC-E2 from these bacteria.

Immunohistochemistry. When HEp2 cells were incubated with dimeric LC5 IgA (Fig. 5A), they stained cytoplasmically in a homogenous pattern, exhibiting a

similar staining pattern to IgA purified from the serum of PBC patients (Fig. 5B). On the other hand, HEp2 cells were not stained by the negative control recombinant IgA (anti-ARS) (Fig. 5C). Similar results were obtained with PD2. Recombinant human LC5 IgA produced notable staining at the apical domain of the small bile duct epithelial cells and, to a lesser extent, the cytoplasm, in a liver section from a patient with PBC (Fig. 6A). Staining of a healthy control liver section as well as PBC kidney, pancreas, and intestine, however, did not result in an apical staining pattern on epithelial cells (Figs. 6B, 6C, 6D, and 6E). Instead, only conventional mitochondrial staining of these tissues was observed. Therefore, the recombinant human LC5 IgA retained the original *in situ* specificity of the parent IgG antibody conferred on it by the inherited v-regions.

Colocalization of Recombinant IgA and PDC-E2 in Epithelial Cells. We stained monolayers of MDCK cells transfected with the pIgR to determine the behavior of our recombinant when taken up by live cells. Colocalization coefficients were calculated in Laserpix by dividing the sum of the intensities of IgA located with PDC-E2 by the total IgA intensity for a Z-series containing a mini-

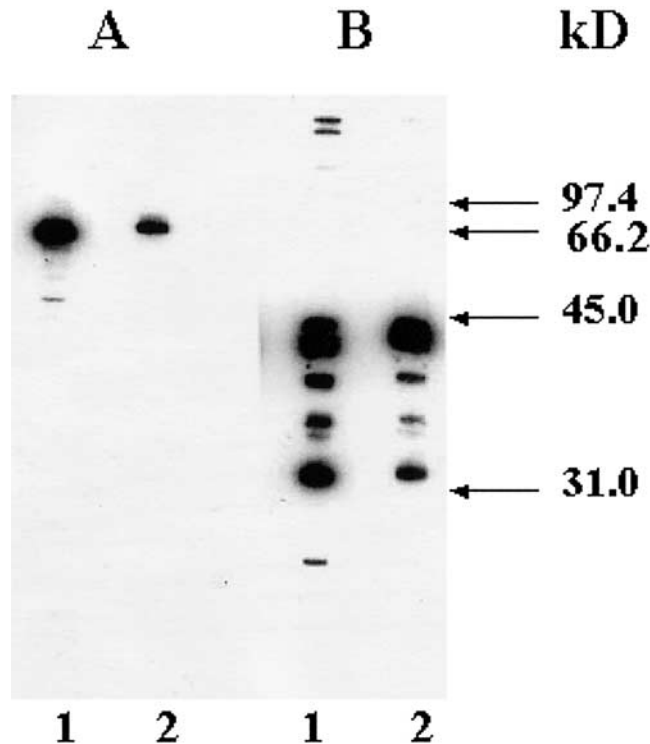


Fig. 4. Reducing 10% SDS-PAGE of (A) beef heart mitochondria and (B) recombinant PDC-E2 probed with PD2 diluted 1:5 in **lane 1** and serum IgA from a PBC patient diluted 1:500 in **lane 2**. In blot A, the band located between 97.4 kd and 66.2 kd corresponds to PDC-E2. In blot B, the PDC-E2 recombinant protein is located at 40 kd; all the smaller bands are the result of abbreviated transcripts and degradation.

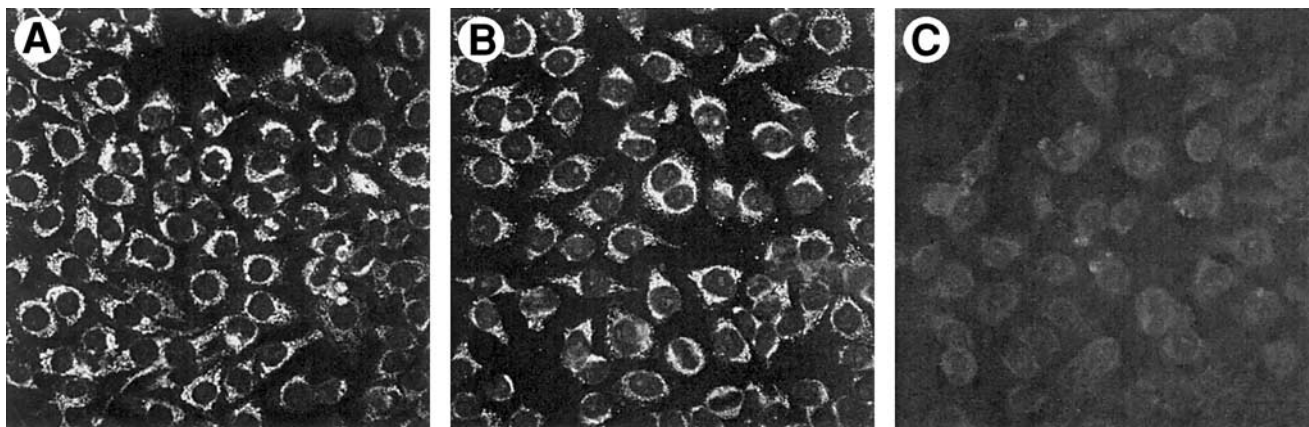


Fig. 5. HEp2 cell staining. Medium containing (A) recombinant dimeric IgA LC5, (B) IgA purified from a PBC patient, or (C) medium containing anti-Ars recombinant IgA was added to commercial HEp2 cell slides. Note that both PBC and recombinant LC5 IgA recognize PDC-E2 in the cytoplasm of HEp2 cells, whereas the control, IgA anti-ARS, does not.

mum of 10 sections ($0.5 \mu\text{m}$). A coefficient of 1.0 represents complete colocalization whereas 0.0 represents none (results are expressed as the mean of 10 IgA expressing cells \pm SEM). Because of the high ratio of dimeric to monomeric IgA (5:1), PD2 was used for this experiment instead of LC5. Dimeric PD2 colocalized strongly with PDC-E2 (0.78 ± 0.035) in the cytoplasm of MDCK-pIgR cells compared with IgA purified from normal sera (0.28 ± 0.017) and myeloma dimeric IgA (0.49 ± 0.041) (Fig. 7). Laserpix colocalization maps of the $0.5\text{-}\mu\text{m}$ Z-series sections of MDCK-pIgR cells treated with recombinant PD2 IgA at $5 \mu\text{g/mL}$ (A) and with control serum IgA at $50 \mu\text{g/mL}$ (B) were generated to visualize the calculated values (Fig. 8). The map depicts the postthreshold image as a combination of binary red (IgA) and binary green (PDC-E2), such that colocalized pixels will appear yellow. Although there are clearly areas on the surface of the cells that are positive for IgA, as well as distinct areas of mitochondrial staining in the cytoplasm, there is extensive colocalization in the cytoplasm of cells treated with PD2 IgA. As was expected, monomeric PD2 (made without the J chain) did not transcytose the MDCK-pIgR cells and therefore we were unable to obtain colocalization data from these cells. In contrast, the MDCK-pIgR cells transcytosed with control serum IgA showed strong IgA staining (red) and good mitochondrial staining (green) with very few areas of colocalization (yellow). Purified IgA from PBC patient sera also was colocalized highly (0.80 ± 0.042) and gave a coefficient almost identical to that of PD2 (Fig. 8). Both dimeric PD2 ($P < .0001$) and PBC IgA ($P < .0001$) were significantly more colocalized with PDC-E2 than control serum IgA and dimeric myeloma IgA1 as determined by the Mann-Whitney test.

Discussion

IgA is the key component of the mucosal immune system, which acts as a first line of defense, protecting the expansive epithelial cell surface area of humans (400 m^2).¹⁸ About 66 mg/kg of IgA is produced daily, which is more than all other isotypes combined.¹⁹ In addition to the ability of IgA to clear microorganisms or toxins on mucosal membranes, an added function of IgA in epithelial cells recently has come into prominence. By binding to the pIgR expressed on the basolateral surface of mucosal epithelial cells, dimeric IgA is transported actively through the cells.²⁰ Several reports have proven that IgA can bind intracellular material, such as newly synthesized viral proteins during transcytosis, via the IgA receptor on the surface of epithelial cells, and neutralize virus.²¹⁻²³ Because the primary target of PBC is the biliary epithelial cell, which is related intimately to the important function of IgA, we have focused with keen interest on the relationship between IgA and PBC, for which the pathogenic mechanism remains unknown. By using fluorescence microscopy, we have reported previously that AMA from PBC patients reacts with molecules present on the surface of biliary epithelial cells (BECs), in the liver of patients with PBC but not normal liver tissues, or in liver tissues from patients with other liver diseases.²⁴ Intense staining of BECs in the livers of PBC patients may reflect the specific internalization of AMA-mitochondrial autoantigen complexes.

Malmborg et al.⁹ showed that serum IgA from PBC patients penetrated epithelial cells in the human IgA receptor-expressing MDCK cell line (MDCK-pIgR) and colocalized with PDC-E2. Although we have not compared the expression level of the pIgR between MDCK

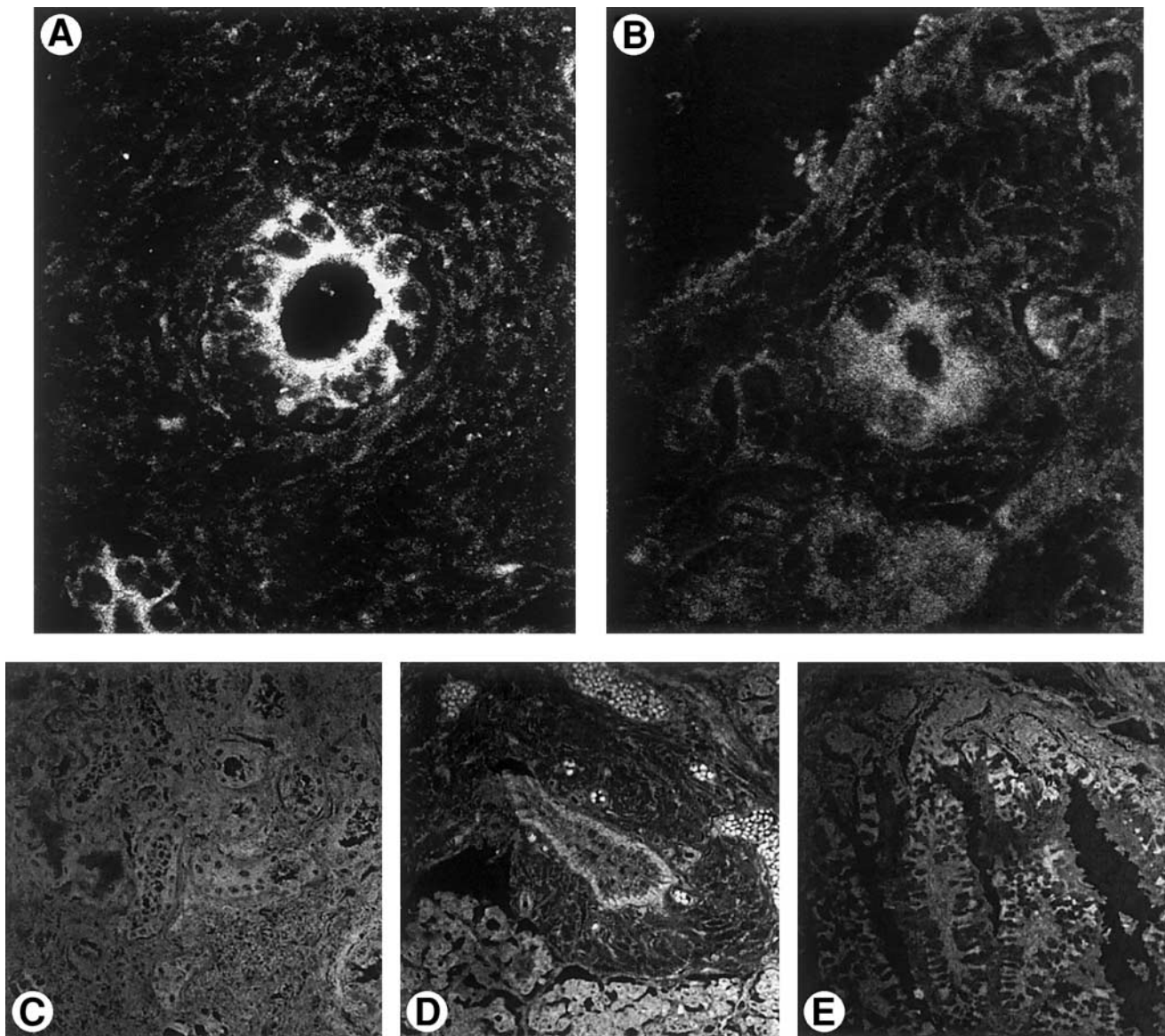


Fig. 6. Liver tissue stained by biotin-labeled LC5 and alkaline phosphatase-conjugated streptavidin-biotin complex. (A) In the liver tissue of a PBC patient, LC5 IgA produces a strong apical staining pattern in contrast to (B) liver tissue from a healthy subject in whom apical staining is extremely faint. (C) Kidney, (D) pancreas, and (E) intestine from PBC patients also failed to show an apical pattern when stained by LC5 IgA. The recombinant LC5 IgA was diluted 1:5 in TBS for the staining of both PBC and control liver sections.

transfectants and human BEC, we believe that this result is not simply a reflection of an unrealistically high expression of the receptor because colocalization also was found in PBC liver tissues. From our colocalization results, we can propose 2 mechanisms that could be responsible for the induction of damage in BECs, both of which might impair mitochondrial function: (1) IgA may target itself to the mitochondria; (2) IgA may bind to nascent PDC-E2 in the cytoplasm during transport to the mitochondria. However, the hypothesis that IgA binds to newly made PDC-E2 outside of the mitochondria is unlikely considering our colocalization findings with the lipoyl acid-dependent PD2 antibody. Given that

lipoyltransferases and lipoylated protein expression is known to be restricted to mitochondria, it is doubtful PD2 would encounter any lipoylated PDC-E2 in the cytoplasm. It is clear that further studies are needed to confirm that PDC-E2 is the identity of the protein bound by IgA and to determine the precise cytosolic location of this colocalization. We also need to consider that because patient antibodies are polyclonal, the immune complex of IgA and PDC-E2 may be carried into the bile duct lumen after binding in the cytoplasm during transport to the mitochondria. The concentration of recombinant IgA that we used per experiment in this study was relatively low. Nonetheless, the fact that we found colocalization of

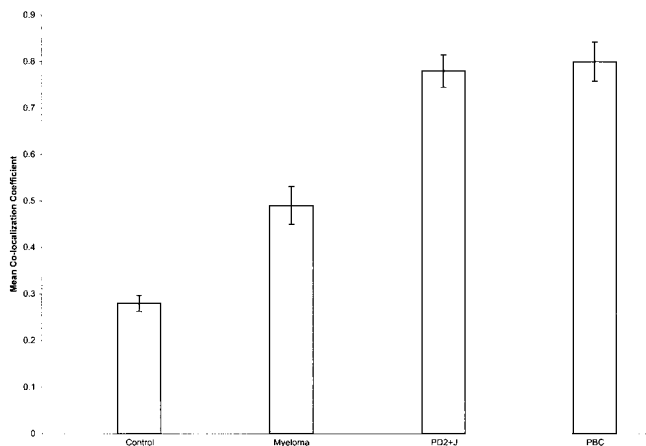


Fig. 7. Data were collected on a Bio-Rad Radiance 2100 laser confocal microscope with a $\times 60$ oil emersion lens. Colocalization analysis was performed with Laserpix v.4.0 software by sampling 10 IgA-positive cells from each image. Recombinant PD2 and PBC patient IgA were significantly colocalized with PDC-E2 ($P < .0001$) compared with control patient IgA as determined by the Mann-Whitney test.

PDC-E2 and the mitochondrial proteins within the MDCK-pIgR cells, suggests that the binding of PDC-E2-specific IgA and mitochondrial proteins in BECs can occur *in vivo*, even if there is only a low abundance of PDC-E2-specific IgA present in bile ducts.

Our hypothesis provides one explanation for an important enigma about PBC, namely, tissue specificity, as this addresses the question of why only epithelial cells are damaged despite the autoantigens for PBC being ubiquitous throughout the human body. There are, nevertheless, some critical questions remaining: How can IgA, which is located in vesicles during transcytosis, recognize and bind autoantigen in the cytoplasm of the cell? Binding and neutralization by IgA after transcytosis into epithelial cells has been reported for some viruses.²¹⁻²³ However, the particles of such viruses exist in the vesicles of the epithelial cells and these particles can meet and bind to IgA by vesicle fusion. Second, why are the small intrahepatic bile ducts of the liver the main target in patients with PBC? Pathology also has been noted within ductal epithelial cells in the pancreas, salivary, and lacrimal glands in PBC patients.²⁵ To answer this question we need to consider the quantity of plasma cells producing anti-PDC-E2 IgA around each ductal organ, the character of the cells, and circumstances that increase or decrease the efficiency of mechanisms including transcytosis, binding of IgA with autoantigen, and mitochondrial dysfunction. Also, we have to consider whether the immunologic manifestations are the primary mechanism or a result of the damage to bile ducts in PBC. There are reports of PBC in patients with selective and severe serum IgA deficiency, although serum IgA deficiency may not correlate

with IgA deficiency in bile.^{26,27} However, compensation by polymeric IgM with J chain, though less efficiently transported across epithelial surfaces compared with IgA, might explain how AMAs can be involved in the pathogenesis for these cases.²⁸ Thus, we have successfully produced 2 recombinant dimeric human IgA proteins specific for PDC-E2 in baculovirus by combining the V_H regions from 2 mAbs with $C\alpha 1$. These antibodies re-

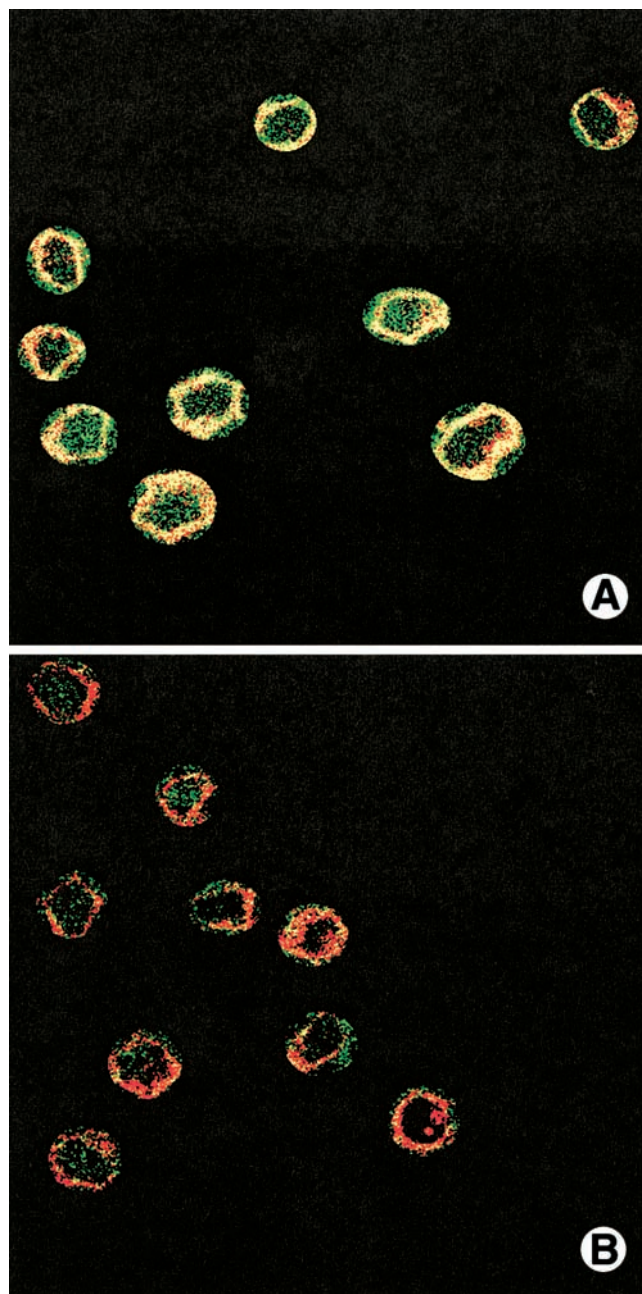


Fig. 8. Laserpix generated colocalization maps of MDCK-pIgR cells treated with (A) recombinant PD2 IgA at $5 \mu\text{g/mL}$ and (B) with control serum IgA at $50 \mu\text{g/mL}$. IgA staining is depicted by the red color; PDC-E2 by the green color; and colocalization by the yellow color. Note the marked difference in colocalization between recombinant PD2 IgA and control serum IgA with PDC-E2 in the cytoplasm.

tained the specificity of those from which the v-regions were derived. Furthermore, this monospecific IgA colocalized with PDC-E2. Although no effects on cell stress or viability were measured, this data suggests that IgA specific for PDC-E2 can contribute to immunopathology.

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