Selective IgA Immune Unresponsiveness to *Proteus mirabilis* Fumarate Reductase A-Chain in Rheumatoid Arthritis

RICHARD H. WEISBART, YOON MIN, ANDREW L. WONG, JOANNE KANG, SUCHADA KWUNYEUN, ANTONY LIN, JOON KIM, GRACE CHAN, RIKA WAKELIN, WILLIAM SOHN, SOPHIA S. CHANG, and ROBERT N. NISHIMURA

ABSTRACT. ***Objective.*** To determine if selective immune unresponsiveness to microbial antigens is associated with predisposition to rheumatoid arthritis (RA).

Methods. Proteins from *Proteus mirabilis* lysate were isolated by SDS-PAGE and examined by Western blotting for antibody responses in sera from patients with RA compared to healthy subjects and patients with psoriatic arthritis (PsA).

Results. Although RA patients had marked IgA immune responses to many *P. mirabilis* proteins compared to healthy subjects, selective unresponsiveness was found in RA to a 66 kDa protein identified as fumarate reductase A-chain (FRD-A) by mass spectroscopy. This was confirmed in Western blots with recombinant FRD-A from *P. mirabilis*. IgA unresponsiveness to FRD-A was found in 21/59 (35.6%) RA patients compared to 7/63 (11.1%) healthy individuals ($p < 0.01$) and 6/52 (11.5%) patients with PsA ($p < 0.01$). IgA unresponsiveness to FRD-A was present in 20/46 (43.5%) RA patients with IgA rheumatoid factors (RF) compared to 1/13 (7.7%) without RF ($p < 0.025$).

Conclusion. Our results identify a selective hole in the IgA immune repertoire for *P. mirabilis* FRD-A in a subset of IgA RF-positive patients with RA. (J Rheumatol 2005;32:1208–12)

Key Indexing Terms:
PROTEUS MIRABILIS RHEUMATOID ARTHRITIS ANTIBODIES
IMMUNE TOLERANCE ANTIBODY RESPONSE

Rheumatoid arthritis is a polyarticular erosive joint disease that affects 1% of the population and is associated with increased morbidity and mortality[1]. The etiology and pathogenesis of RA are poorly understood, but appear to involve genetic predisposition to develop immune mediated inflammation of joints[2]. Certain enteric microbes have been implicated in the pathogenesis of RA, and the microbial intestinal flora of patients with RA was shown to be different from that in healthy individuals[3,4]. These observations are in keeping with the finding that genetic factors influence the composition of the intestinal flora[5-7]. Urinary tract infections with *Proteus mirabilis* occur with increased frequency in patients with RA, and increased levels of antibodies to *P. mirabilis* have been found in the sera of patients with RA[8-17]. Secretory IgA is an important host defense mechanism protecting the gastrointestinal (GI) and urinary tracts, and IgA deficiency is a predisposing factor for the development of many autoimmune diseases[18]. Genetically determined tolerance to self could generate holes in the immune repertoire and might appear as selective IgA unresponsiveness. This immune unresponsiveness to microbial antigens is one of several mechanisms proposed for genetic susceptibility to RA[2]. Thus, we searched for selective IgA immune unresponsiveness to *P. mirabilis* antigens in patients with RA.

From the Departments of Medicine, Veterans Affairs Greater Los Angeles Health Care System, Sepulveda; Department of Medicine, Olive View–UCLA Medical Center, Sylmar; and Department of Neurology, University of California, Los Angeles, California, USA.

R.H. Weisbart, MD; J. Kang, MD; S. Kwunyeun, MD; A. Lin, MD; J. Kim, MD, PhD; G. Chan, BS; R. Wakelin, MS, Veterans Affairs Greater Los Angeles Health Care System; Y. Min, MD; A.L. Wong, MD, Olive View–UCLA Medical Center; W. Sohn, BA; S.S. Chang, BS; R.N. Nishimura, MD, University of California, Los Angeles.

Address reprint requests to Dr. R. Weisbart, VAGLAHS (111S), 16111 Plummer Street, Sepulveda, CA 91343. E-mail: rweisbar@ucla.edu

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MATERIALS AND METHODS

P. mirabilis and *E. coli* were isolated from the urine of a patient with RA and grown overnight in LB medium from a single colony. The bacterial pellets were obtained by centrifugation at 3400 × g for 5 min, washed with phosphate buffered saline (PBS) containing 0.15 M NaCl, pH 7.2, and lysed in B-PER (Pierce Chemical, Rockford, IL, USA) by repeated freezing and thawing.

Patient sera. Venous blood was obtained from 63 healthy adults, 52 patients with psoriatic arthritis (PsA), and 59 adult patients with RA [81% positive for IgM rheumatoid factors (RF)]. Procedures were approved by the Veterans Affairs Institutional Review Board. Serum was separated from whole blood and stored at 4°C until assayed for antibodies to *P. mirabilis* by Western blotting and ELISA.

Measurement of antibodies by Western blotting. Aliquots of *P. mirabilis* lysate containing 20 µg protein were electrophoresed in 4–15% Tris-HCl polyacrylamide gradient gels (4–15% Ready Gel Tris-HCl, BioRad Laboratories, Hercules, CA, USA) in Tris/glycine/sodium dodecyl sulfate (SDS) buffer (Protein Electrophoresis Buffer, BioRad) and transblotted to

nitrocellulose membranes. Protein was visualized by staining with Ponceau S (Sigma, St. Louis, MO, USA), and lanes were excised and incubated overnight with pooled serum diluted 1 in 20 with blocking buffer containing 1% ovalbumin and 0.01% sodium azide from 80 healthy individuals and 80 patients with RA. Nitrocellulose strips were developed with alkaline phosphatase conjugated goat antibodies specific for human IgA and IgG. Alkaline phosphatase was measured by the chromogenic substrate nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate p-toluidine salt in color development solution containing levamisole. In separate experiments, the same method of Western blotting was used to identify IgA antibody responses in the serum of individual subjects to recombinant *P. mirabilis* fumarate reductase A-chain (FRD-A). In those experiments samples of 3 µg protein per lane were evaluated.

Measurement of RF by ELISA. Microtiter plates were coated with Fc fragments from pooled normal serum IgG and incubated with 0.1 ml human serum diluted 1 in 100 with PBS containing 1% ovalbumin. Sera were incubated overnight at 4°C and the plates were washed 3 times with PBS containing 0.05% Tween-20 (PBST). Alkaline phosphatase conjugated goat antibodies specific for human IgA and IgG were incubated in the wells for 1 h. The plates were washed 3 times with PBST and developed with p-nitrophenyl phosphate. The conversion of p-nitrophenyl phosphate to p-nitrophenol was measured by absorption at 405 nm. The mean response ± SD was determined for 63 controls, and positive responses in controls and patients with PsA and RA were defined as those > 2 SD above the mean for healthy controls.

Identification of antigen by mass spectroscopy. A 66 kDa protein was localized in a SDS-polyacrylamide gel stained with GelCode Blue (Figure 1), excised, and identified by lc/ms/ms sequencing on a Micromass Q-Tof hybrid quadrupole/time-of-flight mass spectrometer with nanoelectrospray source by Dr. M.A. Gawinowicz, Columbia University, New York, NY.

Cloning P. mirabilis FRD-A. P. mirabilis FRD-A cDNA was cloned by reverse transcription-polymerase chain reaction (GeneAmp RNA PCR Kit, Applied Biosystems, Branchburg, NJ, USA) from RNA isolated from *P. mirabilis* with an RNeasy Mini Kit (Qiagen, Santa Clarita, CA, USA). Primers were designed based on the 5' and 3' sequence of FRD-A from *Proteus vulgaris* and included 5' *BamHI* and 3' *HindIII* restriction sites. The sense primer was: 5'-GGA TCC CAA ACC TTT AAT GCC GAT ATA G-3', and the antisense primer was: 5'-AAG CTT TCA GCC ATT CGC TTT CTC-3'. The PCR cDNA fragment was ligated into PCR 2.1-topo (Invitrogen, Carlsbad, CA, USA) and sequenced (SeqWright Inc., Houston, TX, USA). The BamHI to HindIII fragment was excised from PCR 2.1-topo and ligated into pQE30 (Qiagen, Valencia, CA, USA). Ligation produced an in-frame fusion with 5' cDNA sequences in pQE30 encoding HIS_6.

Expression and purification of recombinant FRD-A. The fusion protein was expressed in XL-1 Blue (Strategene, La Jolla, CA, USA), purified by binding to Ni-NTA-Agarose (Qiagen, Valencia, CA, USA), washed with binding buffer, and eluted with 250 mM imidazole. Recombinant *P. mirabilis* FRD-A (rFRD-A) was analyzed by SDS-PAGE and Western blotting with antibodies to HIS_6.

Statistical analysis. Antibody responses in Western blots were scored positive or negative. Differences in antibody responses between groups were determined by chi-square analysis.

RESULTS

IgA immune unresponsiveness to a 66 kDa protein. IgA and

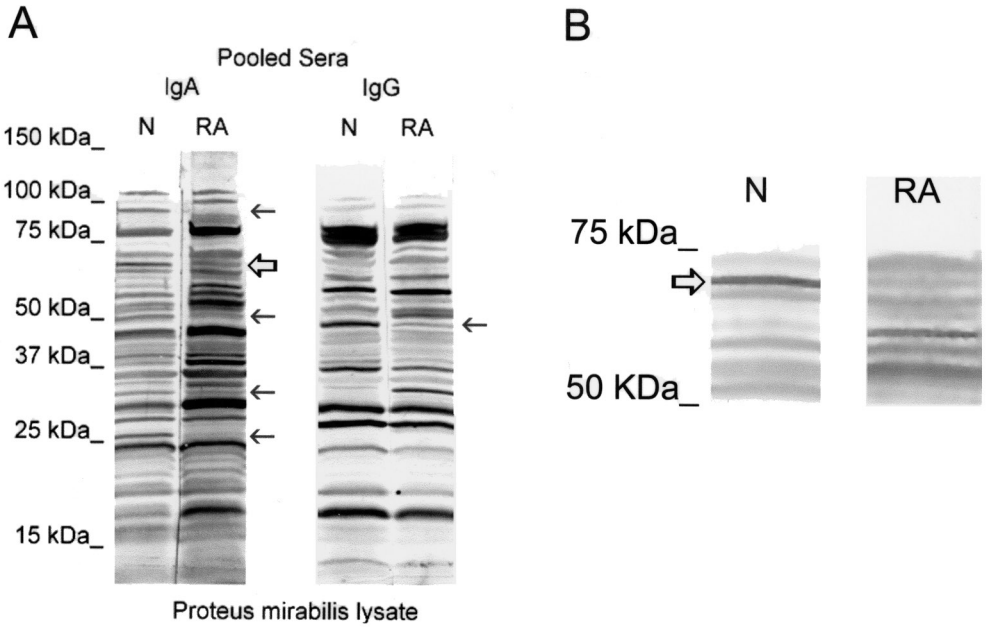

Figure 1. A. IgA immune unresponsiveness to a 66 kDa *P. mirabilis* protein in RA compared to control serum. Serum was pooled from 80 controls and 80 RA patients and assayed at dilutions of 1:20 by Western blotting for binding antigens in lysate from *P. mirabilis*. Elevated levels of IgA antibodies to *P. mirabilis* proteins were predominant in RA sera compared to sera from healthy controls. By contrast, IgG responses were similar in controls and RA. Open arrow indicates a selective decrease in IgA antibodies from RA compared to control sera for binding a 66 kDa protein. Studies of individual sera showed that most control sera contained IgA antibody responses to the 66 kDa protein that were absent in many RA patients. Other candidate proteins showing reduced IgA responses to RA sera include proteins of 26, 30, 50, and 90 kDa (closed arrows). These were excluded from further investigation by studies of individual sera that attributed the results to markedly elevated responses in a few control sera. B. Enlargement showing the 66 kDa protein reactive with pooled control sera compared to RA sera (open arrow).

IgG responses were measured in 80 pooled control and 80 pooled RA sera by Western blotting of *P. mirabilis* lysates electrophoresed in 4–15% polyacrylamide gradient gels in SDS buffer. Elevated levels of IgA antibodies to *P. mirabilis* proteins were predominant in RA sera compared to sera from healthy individuals (Figure 1), consistent with previous reports showing elevated levels of antibodies to *P. mirabilis* in patients with RA. However, IgG responses were similar in controls and RA patients. We looked for immune unresponsiveness to individual proteins in RA patients compared to controls. Several proteins were identified to which RA patients had reduced IgA responses compared to controls, and these corresponded to molecular masses of 26, 30, 50, and 90 kDa (closed arrows, Figure 1) and a 66 kDa protein (open arrow, Figure 1). By contrast, only one reduced IgG response (45 kDa) was identified in RA patients. To determine if responses in pooled sera reflected the prevalence of responses for individual control and RA sera, we assayed multiple individual control and RA sera for IgA responses to each of the candidate proteins. Except for the 66 kDa protein, differences in responses between control and RA sera to each of these proteins were attributable to markedly elevated responses in only a few control sera. The single exception was the 66 kDa protein indicated by the open arrow in Figure 1. Most of the sera from controls had IgA antibodies to the 66 kDa protein, whereas many RA sera did not have IgA antibodies to this protein. Therefore, studies were done to identify the 66 kDa protein and verify differences in control and RA IgA antibody responses with recombinant protein. Screening studies with pooled control and RA sera were also done with lysates from *E. coli*, but selective absence of IgA immune responses in RA sera were not identified (data not shown).

Identification of the 66 kDa protein as FRD-A. The 66 kDa protein was localized in a SDS-PAGE gel stained with GelCode Blue, excised, and identified by lc/ms/ms sequencing. Two peptides from the sequencing (Figure 2) were identical matches to FRD-A previously identified from *P. vulgaris*[19].

FRD-A cDNA from *P. mirabilis* was cloned by RT-PCR from *P. mirabilis* RNA with primers corresponding to the 5' and 3' sequence of FRD-A from *P. vulgaris*. The amino acid sequence of *P. mirabilis* FRD-A was identical to the published sequence of FRD-A from *P. vulgaris* (Figure 2). *P. mirabilis* FRD-A cDNA was ligated into pQE30 for expression in XL-1 Blue (Stratagene) as a fusion protein with an N-terminal HIS_6 tag. Recombinant protein was purified by binding to and elution from Ni-NTA-Agarose. *P. mirabilis* rFRD-A was analyzed by SDS-PAGE and Western blotting and identified with antibodies to HIS_6. The recombinant protein corresponded in size with that identified by a pool of 10 control sera reactive with a 66 kDa protein in *P. mirabilis* lysate but not reactive with a pool of 10 RA sera (Figure 3B). Furthermore, rFRD was reactive with the pooled sera from 10 healthy individuals but not with the sera pooled from 10 RA patients (Figure 3A). These results identified

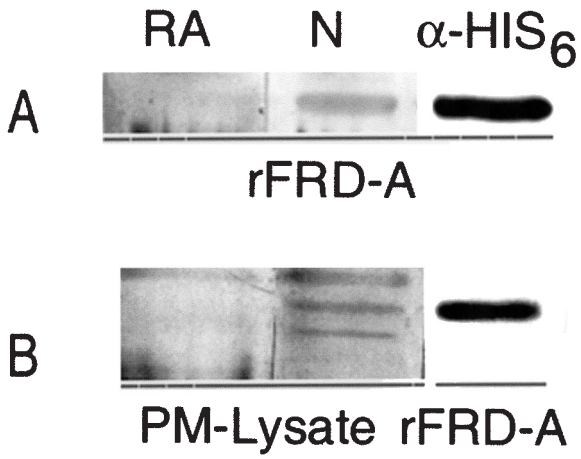

Figure 3. IgA unresponsiveness to rFRD-A in RA. Recombinant HIS_6-tagged FRD-A from *P. mirabilis* was produced to confirm the identity of the protein unresponsive to IgA antibodies in RA sera. Serum was pooled from 10 controls with IgA responses to the 66 kDa protein in *P. mirabilis* lysate and from 10 RA patients without this response. Western blot assays with *P. mirabilis* lysate confirmed that rFRD-A was the same size as the 66 kDa protein reactive with control but not pooled RA serum (B). Pooled control and RA sera showed the same specificity of reactivity in Western blots with rFRD-A, confirming the identity of the 66 kDa protein as FRD-A (A).

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  1  MQTFNADIAIIGAGGAGLRAAIAAAEANPQLKIALISKVYPMRSHTVAAE
 51  GGSAAVTQAHDSYDFHFNDTVSGGDWLCEQDVVDYFVEHCPTEMTQLELW
101  GCPWSRKEDGSVNVRRFGGMKIERTWFAADKTGFHMLHTLFQTSLKYPQI
151  QRFDEHFVLDILVDEGHARGVVAINMMEGTKVQIRANAVIMATGGAGRVY
201  RFNTNGGIVTGDGMGIALRHGVPLRDMEFVQYHPTGLPGSGILMTEGCRG
251  EGGILVNKDGYRYLQDYGLGPETPLGKPENKYMELGPRDKVSQAFWHEWR
301  AGRTIKTHRGDVVHLDLRHLGAKKLHERLPFICELAKAYVGVDPVNEPIP
351  VRPTAHYTMGGIETNQRTETRIKGLFAVGECSSVGLHGANRLGSNSLAEL
401  VVFGRLAGEEAVRRAQEATPANASALDAQTRDIEDNLKKLMNQKGSENWA
451  QIRDEMGEAMEEGCGIYRTPELMQKTIDKLTELKERFKHVEIKDTSSVFN
501  TDLLYKIELGFGLDVAECMAHSAFNRKESRGAHQRLDEGCTERDDVNFLK
551  HTLAFYNPEGAPRLEYSDVKITKSAPAKRVYGGEATAQDKQNKEKANG
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Figure 2. Identification of the 66 kDa protein as FRD-A by mass spectroscopy. The 66 kDa protein was localized in a SDS-PAGE gel stained with GelCode Blue, excised, and identified by lc/ms/ms sequencing. Two peptides (underlined) were identical matches to FRD-A previously identified from *P. vulgaris*.

FRD-A as the 66 kDa protein in *P. mirabilis* lysate preferentially reactive with control compared to RA sera.

IgA unresponsiveness to FRD-A in RA. The prevalence of IgA antibodies in individual RA and control sera was determined in Western blots with *P. mirabilis* rFRD-A. IgA responses were absent in serum from 21 of 59 (35.6%) RA patients. By contrast, IgA responses were absent in only 7 of 63 (11.1%) controls ($p < 0.01$) and 6 of 52 (11.5%) patients with PsA ($p < 0.01$). Representative IgA responses by Western blotting to rFRD-A from 3 positive control and 3 negative RA patients are shown in Figure 4. The purified recombinant protein is shown as the predominant protein stained with GelCode Blue. IgA antibody responses are shown for 3 healthy individuals (subjects 4–6). The absence of IgA responses to rFRD-A is shown for 3 RA patients (patients 1–3). The Western blot is more sensitive than GelCode Blue, so IgA antibody responses are seen to other microbial proteins as well. These responses indicate that individual RA patients make IgA antibody responses to microbial proteins similarly to healthy subjects, and the absence of responses to rFRD-A is selective, and cannot be attributed to immune suppression from therapy or the absence of IgA antibodies. Unresponsiveness to rFRD-A in RA patients was not associated with age, sex, or use of antirheumatic medications (data not shown).

Association of IgA unresponsiveness to FRD-A with seropositive RA. RA is a heterogeneous disease, and serum rheumatoid factors characterize a subset of patients who tend to have more severe and aggressive disease. IgA RF correlate with disease severity and prognosis and tend to be absent in patients with spondyloarthropathies such as PsA[20–22]. Therefore we measured serum IgA RF to determine if IgA unresponsiveness to *P. mirabilis* FRD-A is associated with seropositive RA. IgA RF was present in sera from 9 of 63 (14.3%) healthy individuals, 7 of 52 (13.4%) patients with PsA, and 46 of 59 (78.0%) patients with RA. IgA unresponsiveness to FRD-A was present in 20 of 46 (43.5%) RA patients with IgA RF compared to 1/13 (7.7%) RF-negative RA patients ($p < 0.025$). Although RF can produce false-positive responses, they would not explain selective absence of IgA antibodies to *P. mirabilis* FRD-A-chain in patients with RA. RA is a heterogeneous disease, and IgA unresponsiveness to *P. mirabilis* FRD-A-chain may reflect specificity for a RF-positive subset of patients. The presence of IgA RF in patients with selective absence of IgA antibodies to *P. mirabilis* FRD-A-chain also excludes IgA deficiency as a trivial explanation for absence of IgA antibodies to *P. mirabilis* FRD-A-chain in selected patients.

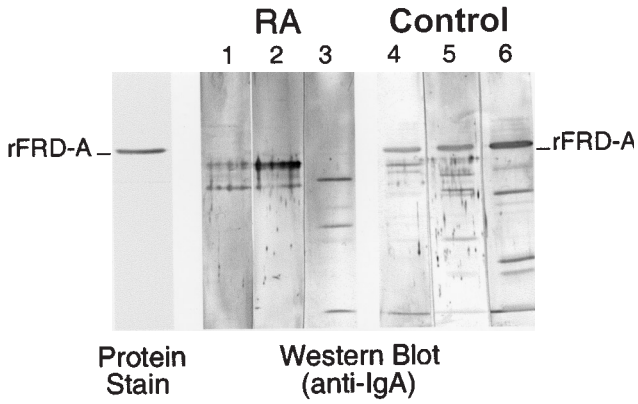

Figure 4. Individual control and RA IgA responses to *P. mirabilis* rFRD. Representative IgA responses to rFRD are shown in Western blots with serum from 3 controls with positive responses (subjects 4–6) and 3 RA patients with negative responses (subjects 1–3). IgA responses in RA patients to other microbial antigens indicate that unresponsiveness to *P. mirabilis* FRD-A is selective and cannot be attributed to immune suppression due to therapy or the absence of IgA antibodies.

DISCUSSION

P. mirabilis is a facultative anaerobe, a member of the family Enterobacteriaceae and the tribe Proteeae. Proteus species are ubiquitous in the environment and are found as part of the normal fecal flora of humans and animals. Immune unresponsiveness to FRD-A would not be expected in RA, since patients with RA have elevated levels of serum antibodies to *P. mirabilis*, and FRD-A is immunogenic. Unresponsiveness to foreign antigens can be the result of tolerance due to homology between self and foreign antigen[23]. We suggest that molecular homology between *P. mirabilis* FRD-A and self may explain immune unresponsiveness to this foreign antigen. Although certain HLA-DR alleles serve as a genetic marker for predisposition to RA, their shared epitope, QKRAA, was not identified in the amino acid sequence of FRD-A[24]. A search of GenBank did not identify homology of FRD-A with other HLA antigens, but homology with other self-antigens is possible, and homology could involve a conformational determinant.

FRD is a membrane-bound iron-sulfur flavoprotein composed of A and B chains that form the catalytic site and a C chain responsible for membrane binding. FRD permits bacteria to grow anaerobically by utilizing fumarate as a terminal oxidant for respiration[25]. In some microbes, FRD may be soluble in the periplasmic space[26]. FRD-A of *H. pylori* is immunogenic, and was shown to be necessary for microbial colonization in the stomach, as confirmed by engineering an *H. pylori* knockout of FRD-A[27,28]. If FRD-A confers the same propensity for *P. mirabilis* to colonize, then the lack of immune reactivity to FRD-A may also translate into colonization of the bacterium in RA. This might explain the increased predisposition for patients with RA to harbor this microbe, as evidenced by an increased prevalence of *P. mirabilis* urinary tract infections in RA patients. Other mechanisms may be necessary, however, to explain inhibition of colonization of the bacterium, since FRD-A is intracellular. It is of interest that proteases produced by *P. mirabilis* degrade IgA[29-32]. There may be multiple mecha-

nisms that predispose to colonization by *P. mirabilis* based on impairment of IgA host defense.

FRD-A, which contains the catalytic domain, is immunogenic, suggesting that it may be exposed to the immune system in the GI tract by senescent or dead microbes[28]. The phagocytic and antigen-processing system of the GI tract has been shown to transport microbial antigens to synovial tissues[33,34]. The absence of antibody to FRD-A could alter antigen processing, predispose to microbial invasion, or influence the transport of various microbial proteins, including FRD-A, through the GI tract. Antibodies to FRD-A could also neutralize enzymatic activity that might condition the intestinal environment as a predisposing factor for RA. Further studies will be required to determine the mechanisms by which IgA unresponsiveness to *P. mirabilis* FRD-A may predispose certain individuals to develop RA.

Additional studies should clarify the possible role of IgA unresponsiveness to FRD-A in the pathogenesis of RA. Our approach to identify selective unresponsiveness of mucosal immunity to microbial antigens may have broad application for future studies to establish the etiology, pathogenesis, and therapy of many autoimmune and infectious diseases.

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