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Autoimmunogenicity of the Helix-Loop-Helix DNA-binding Domain

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ABSTRACT

Nonimmunogenic character of native DNA, and its high immunogenicity when presented in complex with the DNA-binding proteins indicate that the latter might contain molecular triggers of anti-DNA response. To find if this is the case, we have evaluated the autoimmunogenic potential of the main DNA-binding domain of HIV-1 reverse transcriptase that belongs to the canonical helix-loop-helix type. BALB/c mice were immunized with a peptide representing the domain, alone or in complex with the fragmented human DNA in the presence of an adjuvant. Mice were assessed for specific antibodies, autoantibodies against a panel of self-antigens; glomerular immunoglobulin deposition; and for the signs of autoimmune disease, such as proteinuria, and changes in the blood compoments. Immunization with the adjuvanted peptide-DNA complex induced autoantibodies against double-stranded DNA, histones, heterochromatin, and kidney proteins; glomerular IgG and IgA deposition; proteinuria; thrombocytopenia, and anemia. Altogether, this identifies the helix-loop-helix DNA-binding domain as one of the molecular triggers of autoimmunity to DNA and DNA-associated proteins. The experiments cast new light on the role of the DNA-binding retroviral proteins in the induction of autoimmunity, and on the origins of autoimmune complications in the microbial infections in general. It also implies that choosing the DNA-binding proteins as vaccine candidates should be done with precaution.

Key words: DNA-binding domain; reverse transcriptase; anti-DNA autoantibody; heterochromatin; histones; proteinuria; nephropathy; thrombocytopenia.

INTRODUCTION

An autoimmune response against DNA is a hallmark of systemic lupus erythematosus (SLE) and is notable also in rheumatoid arthritis, Sjögren´s syndrom and other autoimmune diseases. Molecular targets of anti-DNA antibodies are multiple with numbers still increasing (Criscione and Pisetsky, 2003), while the molecular mechanisms of initiation and expansion, specifically the molecular triggers of the latter, remain largely unknown. Nonimmunogenic character of eukaryotic DNA free from the adjuvants and carriers (Pisetsky, 1997) indicates that triggers of autoimmunity lay elsewhere, among charged carbohydrates, proteins, or nucleic acid-protein complexes. Indeed, one of the first anti-DNA antibody (anti-DNA) triggers found was a 10-mer peptide that induced severe renal disease in non-autoimmune mice (Deocharan et al., 2002). The capacity of peptides/proteins to induce anti-DNA antibodies was lately attributed to their similarity to DNA backbone, mimicking the hydrogen bonds of the nucleotide bases and/or of bending and twisting of the DNA double helix (Dryden and Tock, 2006). Anti-double stranded DNA antibodies were also generated in response to the bacterial DNA and polysaccharides (Madaio et al., 1984; Desai et al., 1993; Gilkeson et al., 1993; Ray et al., 1996), and through the idiotypic network as a response to antibodies generated to microorganisms (Wun et al., 2001).

The autoimmune response against the native mammalian DNA was mainly achieved after immunization with DNA in complex with the DNA-binding proteins/peptides, such proteins alone (Cerutti et al., 2005; Desai et al., 1993; Moens et al., 2002), ribonucleoproteins, nuclear antigens, and nucleosomes derived from the apoptotic cells (Bach et al., 1998; Isenberg et al., 1997; van Venrooij and Pruijn, 1995) (Desai and Marion, 2000) (Voynova et al., 2005). Relevance of the strong autoimmunogenicity of the latter to the disease is supported by coexistence of anti-DNA and antinucleosome antibodies in scleroderma, mixed connective tissue

diseases, and lupus, the latter manifested by aberrations in apoptosis and reduced clearance of apoptotic cells (Amoura et al., 2000; Berden, 2003).

Autoimmunogenicity of the DNA-binding proteins indicated that they may contain the molecular triggers of autoimmune response to DNA, as is the case for ribonucleoproteins (RNP). Monneaux & Muller had found that the autoimmunity to RNPs could be initiated by immune response to a peptide trigger, an RNA-binding domain of type 1 (RNP1) (Monneaux and Muller, 2002). Immune response to the proteins bearing this domain led to autoimmunity to a vast panel of RNPs, as core small nuclear RNPs, heterogenous nuclear RNPs, and heterogenous Ro/La antigenic complex (Monneaux and Muller, 2002). Similarly, the DNA-binding motives may initiate an immune response that would spread to bound DNA, the latter acting as an autoadjuvant, i.e. they have to be highly immunogenic. This appears to be the case. Break of tolerance to DNA was shown in response to the peptides from the ubiquitin-fusion protein of *T. cruzi* (Desai et al., 1993); polyoma virus large T-antigen (Moens et al., 2002); HPV16 E2 (Cerutti et al., 2005). All three have a common feature – they contain the canonical zinc finger DNA-binding motif (http://www.expasy.ch/cgi-bin/prosite-search-ac?PS00028).

We hypothesized that high immunogenicity and capacity to initiate immune response spreading to DNA may be an inherent feature of not only the canonical zinc finger, but also of other DNA-binding motives. To test this, we choose the α-helix-turn-α-helix motif (HTH) of the homeobox, widely distributed in the DNA-binding proteins (Wintjens and Rooman, 1996). There is an experimental evidence of high immunogenicity of oligopeptides incorporating this motif (Kashuba et al., 2006) with frequent localization of B-cell epitopes in the proximity (Chiba et al., 1996; Yi et al., 2000). HTH is the main DNA-binding motif of reverse transcriptases (RT), highly conserved among retroviral Pol polypeptides including HIV-1, HIV-2 and SIV (Hermann et al., 1994; Beard et al., 1996). Two alpha-helices of HTH function as “tracks” on which the

template-primer moves during translocation, determining enzyme fidelity, correct positioning, and precise RNAse H cleavage (Beard et al., 1996; Jacobo-Molina et al., 1993; Powell et al., 1999). The HTH region of HIV-1 RT is immunogenic inducing conformational antibodies that inhibit the RT activity (Chiba et al., 1996; Ohba et al., 2001). We have indications of possible HTH autoimmunogenicity, as we have earlier shown that immunization with wild-type HIV-1 RT leads to induction of antinuclear and anti-DNA antibodies (Isaguliants et al., 2004), while immunization with RT variants rigorously missfolded and devoid of polymerase activity induce neither autoimmune response, nor pathology (Rollman et al., 2005; Starodubova et al., 2008a; Starodubova et al., 2008b; Isaguliants M, Petrakova N unpublished).

Here, we have shown that immunization of mice with double-stranded DNA complexed to a peptide representing the HTH domain of HIV-1 RT can break tolerance to DNA with spreading of autoimmune response to heterochromatin, histones and kidney proteins, leading to systemic autoimmune disease. Altogether, this identifies the helix-loop-helix DNA-binding domain as one of the molecular triggers of autoimmunity to DNA.

MATERIALS AND METHODS

DNA, oligo- and polypeptides

Oligopeptide VNDIQKLVGKLNWASQIYPGIKVRQLCKKLLRTKALT (RT-P) corresponding to aa 254-290 of reverse transcriptase (RT) of HIV-1 LAI was synthesized by Thermo Electron GmbH (Ulm, Germany). HIV-1 RT protein expressed in *E. coli* and purified by affinity chromatography was a kind gift of Dr Kochetkov (WA Engelhardt Institute of Molecular Biology, Moscow). Rat heterochromatin, constituted mainly by the nucleosome particles, was prepared from the homogenized rat liver as was described earlier (Li et al., 1999). The sources of other autoantigens were as follows: single-stranded human placental fragmented DNA (ssDNA),

double-stranded (ds) human placental DNA, cytoplasmic, membrane and nuclear protein extracts from mouse kidney tissues, from Sigma (St. Louis, MO); DNA/RNA-free histones from calf thymus, from US Biological (Swampscott, MA); small nuclear ribonuclear protein (RNP or Sm) comprised of RNP 68K and SmD polypeptides, Ro (SSA), and La (SSB) from calf thymus, from The Binding Site (Birmingham, UK). Nuclear, cytoplasmic, and membrane kidney protein extracts used in ELISA for assessing anti-kidney antibodies were from Sigma.

Immunogens

Immunogens were RT-P, fragmented dsDNA, or fragmented dsDNA complexed to RT-P (RT-P/DNA). Fragmented dsDNA was prepared by shearing native human placental DNA to 500-2000 base pairs (bp) by ultrasonication. Fragmented DNA was complexed to RT-P by mixing their solutions at initially high ionic strength with gradual decrease of the ionic strength to physiological by step-wise dilution as was described for the chromatin reconstitution (Li et al., 1999). RT-P and DNA were taken in a 25:1 molar ration optimal for the formation of dsDNA-peptide complexes (Desai et al., 1993; Desai and Marion, 2000). The assays for RT-P/DNA binding were performed according to the procedures described earlier (Desai and Marion, 2000).

Mouse Immunization

The study was carried in compliance with the International Guiding Principles for Biomedical research Involving Animals and was approved by the local authorities in lines with national regulations. Groups of 6 to 12 twenty-week old BALB/c mice (Manikhino Experimental Farm, Moscow region, Russia) were immunized five times with RT-P, or DNA, or RT-P complexed to DNA using a low-dose immunization protocol (Jeannin et al., 1993; Kashuba et al., 2006). Mice were primed on weeks 1 and 2, and boosted, on week 3, 7, and 11. The dose of RT-P in priming was 15, and in boosts, 7.5 μg; and the dose of DNA, 100 and 50 μg, respectively. Immunogens were administered in PBS with either the complete (CFA), or incomplete (IFA) Freund´s

adjuvant in primes, IFA in boosts, or in PBS without the adjuvant. The ratio of immunogen to the adjuvant was 1 : 1 to 1.5 (v/v). Immunization mixture was administered i.m. in the right hind leg (100 μl) and i.p. (200 μl). Control mice were mock-immunized with PBS with or without adjuvant, or left untreated.

Blood and urine analysis

The effects of immunization were assessed by regular examination (Table 1). Urine was collected weekly. Blood was collected from the retroorbital vein plexi prior to the immunization, and on weeks 5, 9, and 13. Urine samples were analysed using urine test strips with color charts for protein, glucose, and pH (TriPHAN®, PLIVA Lachema Diagnostika, Brno, Czech Republic). There was no difference between groups in the urine positivity to ketones, pH, or glucose content (all <6 mmol/l). Protein content in the urine samples collected at week 13 was also analysed by the Bradford method run in triplicates (Bio-Rad protein assay, BioRad, Munchen, Germany). Blood collected at week 13 was analysed on an automated haematology analyzer Hema Screen 18 (Hospitex Diagnostics, Sesto Fiorentino, Italy). Otherwise, blood smears were prepared, stained according to the May-Grunewald-Giemsa method and examined microscopically.

Pathology evaluation

On week 13, all mice were sacrificed and the necroscopy was performed as described (http://icg.cpmc.columbia.edu/cattoretti/Protocol/mousepathology/mousetissue.html#routine). One kidney from each mouse was frozen in the liquid nitrogen and kept at –70°C. Other organs (spleen, liver, lungs, brain, heart, quadriceps at the site of injection, and the other kidney) were collected and fixed in the 10% neutral-buffered formalin. After 18 h at +8°C, fixed tissues and organs were washed with the excess of PBS, and embedded in paraffin. Paraffinized fixed tissues were cut by microtome into 4 μm sections, mounted on glass slides, and stained by the routine

H&E technique. Histology was evaluated microscopically by an experienced pathologist without knowledge of the treatment assignment. Histopathological changes in the renal tissue were reported as described earlier (Putterman et al., 1996).

Assessment of immunoglobulin deposition in kidneys

Sets of 5 μm sections of frozen kidneys were prepared by the Histology facility, Microbiology and Tumorbiology Center, Karolinska Institutet, Sweden. Slides were blocked in Scan-buffer (PBS supplemented with 0.5% BSA, 2% normal goat serum, 0.05% Tween 20 and 0.01% merthiolate) for 1 h at 20°C, and double stained first with rabbit anti-collagen IV serum (Progen, Germany) for 1 h at 37°C, and then for 1 h at 37°C with FITC-conjugated goat anti-mouse Ig and TRITC-conjugated goat anti-rabbit Ig (both Dako Denmark A/S, Glostrup, Denmark). Nuclear staining was done with 5 μM DAPI (4.6'-diamidino-2-phenylindole hydrochloride). Rinsed slides were mounted in the Vectashield mounting medium (Vector Laboratories, CA, USA) and evaluated on a fluorescence microscope (Olympus BX60; Tokyo, Japan) with Olympus 40×/0.85 or Olympus 20×/0.50 ocular objectives with the attached Sony 3ccd camera DKC-500 and Pentium PC computer equipped with the AFG VISION*plus*-AT frame grabber board. Anti-collagen-IV antibodies and DAPI visualized all glomeruli, and anti-mouse-FITC, some. Percentage of glomeruli containing mouse Ig deposits was calculated by dividing the mean number of glomeruli stained by anti-mouse antibodies by the mean total number of glomeruli as assessed by DAPI and anti-Col IV-immunostaining. The latter incorporated readings from 3 slides per mouse with 5 fields per each slide. Variation in the total glomeruli numbers between slides made from one kidney was <5%, and between DAPI- and anti-ColIV based methods of glomeruli counting, <10% (data not shown). Subtyping of deposited mouse Ig was done by staining of dewaxed paraffin sections using goat anti-mouse IgG, IgA, or IgM labeled with

horseradish peroxidase (HRP) (www.labvision.com/pdf/Immunochemistry.pdf). Slides were viewed on a light microscope at x10 and x20 magnification.

Serology

Indirect ELISAs were performed on the MaxiSorb 96-well plates (Nunc, Denmark). Coating with peptide RT-P was performed with 100 μg/ml solution in 0.05 M carbonate buffer, pH9.3, for 20 h at 20°C, followed by 3 days at 6-8°C. Proteins (RT, histones, RNP, La, Ro60, kidney protein extracts) and heterochromatin were coated as 0.2 μg/ml solutions in PBS, pH 7.3, for 16 h at 6-8°C. Anti-cardiolipin antibodies were assessed on the MaxiSorb 96-well plates coated with cardiolipin (Sigma) by evaporating its 5 μg/ml solution in ethanol overnight at 4°C (Rupin et al., 1991). ELISA was performed as described earlier (Isaguliants et al., 2000). TMB (Medico-Diagnostic Laboratory, Moscow, Russia) was used as a substrate. Positive controls were taken from the mouse anti-histone antibody kit (Alpha Diagnostics Int/ADI, TX, USA). The cut-off level for specific absorbance at each serum dilution was determined as values exceeding the mean specific absorbance of 11 naïve mouse sera assessed at this dilution plus 3 standard deviations (SD). Titers of antibody-positive sera for each antigen were determined. Each mouse was then represented by the maximal titers, i.e. the highest reached throughout the immunization. Specific character of antibody binding to RT-P and RT was assessed by a free peptide competition assay as described earlier (Steward et al., 1991). Autoantibodies against nucleus (ANA), native ribonuclear protein (nRNP), and circulating immune complexes (CIC) and also histones and heterochromatin were detected using respective ADI kits. The commercial and in-house assays for histones and heterochromatin gave concordant results (data not shown).

Detection of anti-DNA antibodies

Autoantibodies to DNA were assessed by ELISA using as a substrate double-stranded human

DNA (nDNA) (Janyapoon et al., 2005), and single-stranded DNA (ssDNA), both from Sigma. ELISA of anti-DNA antibodies were performed on the 96-well Universal Covalent microtiter plates (Corning Costar Corporation, Cambridge, MA) coated with human dsDNA (nDNA) or ssDNA as was described earlier (Isaguliants et al., 2004). Anti-DNA antibodies were also evaluated by immunofluorescence microscopy using slides with stabilized *Crithidia luciliae* (Fluorescent nDNA test system; Immuno Concepts, Sacramento, CA) as recommended by the manufacturer except for the secondary antibodies were FITC-labelled goat anti-mouse Ig (Dako).

Software and statistics

Immunofluorescent images were processed using Hipic3.2.0 (Hamamatsu, Japan), Image-Pro Plus (Media Cybernetics, Silver Spring, USA), and Adobe Photoshop software. Continuous data sets were evaluated for normality using the Shapiro-Wilk's W test. Comparisons between data sets fulfilling the criteria of normality were done using independent *t*-tests by groups. Other data sets were assessed using nonparametric methods: Kruskall-Wallis test for multiple independent groups, the Mann-Whitney U-test for pair-wise comparisons, and correlations, using Spearman rank R test, and multiple regression analysis. The criterion for accepting statistical significance was $p<0.05$. Statistical analysis was done using STATISTICA AXA 8 (Tulsa, OK, USA).

RESULTS

Set-up of the mouse study: the immunogens and immunization targets

A peptide corresponding to aa 254-290 of HIV-1 RT (RT-P) representing its HTH DNA-binding domain (Hermann et al., 1994) was used as an immunogen alone or in complex with the fragmented double-stranded DNA (RT-P/DNA). Sequence unspecific binding of RT-P to DNA (as anticipated from (Lin et al., 2000)) was achieved by the gradual decrease of ionic strength of the solution bringing it to physiological. Targets for autoimmunity induction were 20-week old female BALB/C mice. Choice of a mouse strain was motivated by the capacity of the BALB/C

strain for high antibody production (Mosmann and Coffman, 1989); and choice of age, by the enhanced production of autoantibodies in the aging animals (Eaton-Bassiri et al., 2000; Goidl et al., 1981). Female sex was assumed to increase the chances of the autoimmune disease development (Pisetsky and Jiang, 2006). Mice were immunized with either RT-P/DNA, or single components of the mixture with or without the Freund´s adjuvant, or with adjuvant alone, or mock-immunized with PBS. The effects of immunization were assessed by urine and blood analysis, survey of gross and micropathology, immune histopathology and ELISA for specific and auto- antibodies (Tables 1, 2).

Antibody response against reverse transcriptase

Screening of mouse sera for specific antibodies revealed that RT-P was highly immunogenic (Fig. 1A). An important factor enhancing the anti-RT-P response was the RT-P/DNA complex formation. In mice receiving the RT-P/DNA complex, the titer of antibodies against RT-P (anti-RT-P) was higher than in mice receiving RT-P alone (both adjuvanted; for RT-P/DNA/CFA vs RT-P/CFA, $p=0.012$), although there was no difference in the frequency of positivity for anti-RT-P (Fig. 1; Table 2). Without Freund´s adjuvant, the RT-P/DNA complex induced a specific response in 30% animals (2/6; titers 100 and 15000; Fig. 1A; Table 2). The adjuvant significantly enhanced the magnitude and frequency of anti-peptide response, with seroconversion to anti-RT-P positivity reaching 100% and titers exceeding 10^5 (Fig. 1A; Table 2). Low titer anti-RT-P were detected also in one adjuvant-immunized animal (#39; Table 2). No peptide-specific antibodies were found in naïve, or mock-immunized mice (Fig. 1A; Table 2**).**

Immunization with RT-P/DNA led to the induction of antibodies recognizing reverse transcriptase (anti-RT). Anti-RT were formed in totally 70% (17/24) of mice receiving RT-P/DNA, and one mouse from the RT-P/CFA group (Table 2). Frequency and levels of anti-RT were similar for all groups receiving RT-P/DNA complex (either with or without the adjuvant;

p>0,05). Out of 17 RT-positive mice, 13 were also positive for anti-RT-P. The titers of anti-RT-P and anti-RT were, however, not correlated. More so, while RT in solution competed for antibodies with RT on the solid phase, free RT-P did not inhibit serum binding to RT (data not shown). Anti-protein RT antibodies were also induced in some of the control mice (11/40; 27.5%). Control groups did not differ in either levels, or frequency of anti-RT antibodies ($p > 0.05$), both were significantly lower than the respective values in mice receiving RT-P/DNA ($p<0.05$; Fig. 1A; Table 2).

Autoantibody response against DNA

Mice receiving adjuvanted RT-P/DNA developed high titers of antibodies against nDNA (anti-nDNA; average titer >1500; Fig. 1B). Selected sera with anti-dsDNA titer >500 tested positive for anti-dsDNA in the Crithidia test (data not shown). Complete and incomplete Freund´s adjuvants were equally efficient in promoting anti-DNA induction. Anti-nDNA levels in all other groups were significantly lower ($p<0.05$; Fig. 1B). Peptide aided to development of strong anti-DNA response, as RT-P/DNA/CFA-immunized mice demonstrated a significantly higher frequency ($p<0.05$; T-test) and a tendency to a higher level of anti-nDNA than mice receiving DNA/CFA ($p=0.1$). The levels of both anti-RT-P and anti-RT correlated to the levels of anti-nDNA (p values <0.0005). Mice positive for both anti-RT protein and anti-RT-P antibodies had significantly higher anti-nDNA titer than mice positive for a single antibody type (1600 compared to 600; Fig. 2A). Anti-RT-P titer also determined the levels of anti-nDNA in the multiple regression analysis ($p<0.001$). Induction of anti single-stranded (ss) DNA was not specific (Fig. 1B; Table 2), however, mice double positive for anti-RT-P and anti-RT had higher titers of anti-ssDNA antibodies (Fig. 2B).

Autoantibody response against nuclear components

RT-P complexed to DNA induced anti-heterochromatin antibodies (Table 2). Their maximal

titers highly correlated to the maximal titers of antibodies against RT-P and nDNA (p values <0,003). RT-P, DNA, and their complex administered in CFA (but not in IFA) triggered autoantibody response to histones. There latter did not depend of RT-P/DNA complex formation, as there was no difference in the incidence of anti-histone antibodies between RT-P/DNA/CFA and DNA/CFA groups (Table 2). CFA was insufficient to raise anti-histone antibodies if taken alone. Anti-histone antibodies correlated to the presence and titer of antibodies against nDNA and anti-heterochromatin (p values < 0.0005) but not to anti-RT-P or anti-RT. Mice receiving RT-P/DNA/CFA were significantly more often positive for ANA than mice in all other groups ($p<0.05$). On the overall, antibody positivity for nuclear-derived antibodies decreased in the row RT-P/DNA/CFA >>RT-P/DNA/IFA>RT-P/DNA>RT-P/CFA≈DNA/CFA≈CFA/IFA (Table 2). Presence of ANA correlated to the presence of antibodies against RT-P, RT, nDNA, and heterochromatin (p values <0,01), but not those against histones (Table 2).

Autoantibody response to kidney

Protein targets of the autoantibodies to kidney were assessed by ELISA utilizing nuclear, cytoplasmic, and membrane kidney protein fractions (in analogy to (Bernstein et al., 1993)).

The induction of antibodies against kidney nuclear protein fraction strictly required the RT-P/DNA complex formation and adjuvant aid (Fig. 3; Table 2) and highly correlated to the presence of autoantibodies to heterochromatin, and histones (p values $<10^{-5}$). Infrequent anticytoplasmic antibodies were apparently due to the introduction of DNA in CFA, however, complexing DNA to RT-P enhanced their formation almost 10-fold (3600±3000 as compared to 458±500, respectively; Fig. 3; Table 2). Anti-kidney membrane proteins were induced in all CFA recipients (Table 2). Their correlation to the levels of anti-kidney cytoplasmic and anti-histone antibodies (p values <0.005) indicated that the reactivity was directed against proteins. A

common feature of all types of autoreactivity to kidneys was its correlation to the autoantibody response to histones ($p<10^{-5}$), but not to RT-P, RT, or DNA.

Overall frequency of autoantibody induction

All mice receiving RT-P/DNA (n=24) had a significantly higher frequency of autoantibodies than the controls (n=46; $p<10^{-5}$; Table 2), specifically what concerns the autoantibodies against nDNA ($p<0.00005$), heterochromatin ($p<0.02$), and ANA ($p<0.05$). The average number of autoimmune reactions in the RT-P/DNA/CFA group was significantly higher than in all other groups ($p<0.05$). In this group, all mice had autoantibodies against at least one, and a mean of four autoantigens (Table 2). No difference was revealed in the levels of circulating immune complexes (CIC), and antibody reactivity to RNPs, cardiolipin, or BSA control (data not shown).

Renal deposition of immunoglobulins

Immunostaining of frozen sections visualized deposition of antibodies in kidneys and determined their subclasses (Fig. 4, 5). Immunoglobulin deposits were mainly found in the kidneys of mice receiving adjuvanted RT-P (RT-P/DNA/CFA, RT-P/CFA); DNA has no additional effect on the deposition (Fig. 6). Deposits were also found in several mice immunized with DNA/CFA. No immunoglobulin deposits were found in mice that received RT-P without CFA (RT-P/DNA/IFA, or RT-P/DNA), in mock-immunized mice, or mice immunized with the adjuvants (Fig. 4, 6). Presence of the deposits correlated with the presence of autoantibodies to histones and to DNA in high titer (reflected by positivity in the Crithidia test); the level of deposition was significantly higher in anti-nDNA positive than in anti-nDNA negative animals (p values <0.05).

Deposits were localized in the glomeruli generating a characteristic pattern of mesangial staining, not involving the glomerular basement membrane and were composed of mouse IgG and IgA; IgM were not detected (Fig 4 A-C; Fig 5, and data not shown). Interestingly, similar

pattern of immunostaining was observed when staining kidney sections of normal BALB/c mice with pooled sera of mice receiving RT-P/DNA/CFA, but not of mice receiving CFA/IFA, or of the mock-immunized controls (data not shown).

Pathology and kidney function

Mice immunized with RT-P/DNA/CFA had visual signs of autoimmune disease as hair loss, ascites, and liver fibrosis (otherwise detected only in one mouse receiving CFA; Table 1; and data not shown). Two mice in this group died during immunization. Comparison of mice receiving RT-P/CFA, or DNA/CFA, or CFA alone to RT-P/DNA/CFA recipients revealed that that the latter had significantly larger spleens with pronounced fibrosis; spleen weight increased 2.5-times compared to that in the RT-P/DNA-immunized, and 2-times compared to that in the DNA/CFA- or CFA/IFA-immunized animals (Table 1). Pathomorphological examination of haematoxylin and eosin stained kidney sections demonstrated different grades of glomerular sclerosis and fibrosis without marked inflammation. RT-P, DNA, and RT-P/DNA-immunized mice showed signs of mild sclerosis affecting 30 % of the animals.

Major differences were revealed by the qualitative (by Uristrip) and quantitative analysis of the urine for protein content. By the end of experiment, mice receiving adjuvanted RT-P with or without DNA had significantly higher levels of protein in urine than mice in all other groups ($p<0.01$; Fig. 7). More so, mice positive for anti-RT-P antibodies had significantly higher proteinuria than seronegative animals ($p<0.05$; data not shown). In mice immunized with RT-P/DNA/CFA, proteinuria developed earlier than in mice receiving RT-P/CFA (Fig. 7).

Haematology findings

Populations of white blood cells (WBC) as a whole, neutrophil bands and eosinophils did not differ. Adjuvanted RT-P and DNA were toxic for RBC (Table 1; and data not shown). All mice

receiving RT-P, DNA and CFA in different combinations had significantly lower counts of red blood cells (RBC; 3.03±0.45 x 10^6/μl) than the controls (5 to 15 x 10^6/μl). Mice positive for anti-RT-P had lower RBC counts, and more pronounced difference in erythrocyte size (anisocytosis), poikilocytosis, and microscopically observed changes in the hemoglobin content in single red blood cells (polychromasia) ($p<0.05$; Table 1). All parameters were associated to the presence of autoantibodies against DNA (p values <0.05).

Significant deviations were observed in the platelet counts and shape (Table 1, Fig. 8). All mouse groups except that receiving RT-P/DNA/CFA had mean platelet counts (PLT) of 320000 to 370000 per mm^3, whilst in mice receiving RT-P/DNA/CFA their numbers were reduced to 210000 per mm^3 (Fig 8). These mice had also lower mean platelet volume (MPV). The decreased MPV correlated to spleen and liver fibrosis, and splenomegaly (p values <0.05). Strong inverse correlations were observed between PLT, MPV, and proteinuria (all p values <0,05). Cumulative disturbances in megakaryopoiesis determined proteinuria in multiple regression analysis. Low PLT and MPV correlated with the presence and titer of antibodies against RT, RT-P, and heterochromatin (but not anti-DNA); anti-heterochromatin antibodies were also a predictive of low MPV values in the multiple regression analysis (p values <0.05).

DISCUSSION

To study the autoimmunogenicity of a single DNA-binding motif, we have selected a helix-turn-helix domain of HIV-1 reverse transcriptase (RT-P; Hermann et al., 1994) and used it as a peptide immunogen, either alone or in complex with native DNA. Peptide was immunogenic by itslef. However, complexing to DNA led to significant enhancement of its immunogenicity supporting the concept of high immunogenic potential of the DNA-binding peptides and proteins. The additive effect of the peptide and DNA components of the complex both contributed to the break of tolerance to DNA was clearly demonstrated, as mice receiving

adjuvanted RT-P/DNA developed anti-DNA autoantibodies in high titer and with high frequency (90 to 100%). Autoimmune response was augmented by the adjuvant that provided a background immune stimulation/polyclonal B-cell stimulation enhancing the production of both specific and auto- antibodies.

The induction of anti-DNA response by the complex may have been initiated by the way RT-P binds DNA. Binding of DNA by RT HTH leads to a distortion of the DNA-duplex seen as a transition from the B- to A-form with a sharp bend of about 41 degrees that occurs over the four base pairs (bps) between the A-form at first five bps near the polymerase active site, and the B-form at the last nine bps towards the RNAse H active site (Ding et al., 1997). A wide variety of DNA sequences potentiates the transitions from the B- to non-B form DNA structures (Ban et al., 1994; Cox and Mirkin, 1997)**.** Such distorted non B-form DNA-duplexes were shown to be autoimmunogenic (Klinman, 1996; Pisetsky and Reich, 1999; Sanford et al., 1988; Stollar, 1986; Zarling et al., 1984). This may explain the autoimmunogenicity of HTH and other DNA-binding motives: they may ease the break of tolerance to DNA by presenting it in the non-B form.

The break of tolerance expanded beyond the induction of anti-DNA antibodies. Mice receiving RT-P complexed to DNA formed also antibodies to heterochromatin and histones. Formation of anti-heterochromatin antibodies as well as of ANA required immunization with DNA bound to RT-P that could not break the tolerance to DNA if taken alone. On contrary, this complex was not required for the induction of anti-histone antibodies. Their levels correlated only with the levels of autoantibodies to DNA and heterochromatin indicating that they might have been formed as a result of epitope spreading from RT-P to DNA and further. Another indication of epitope spreading were the autoantibodies to kidney that highly correlated with that against heterochromatin and histones. Thus, the administration of RT-P/DNA triggered the induction of autoantibodies against a number of self-antigens with indications of epitope spreading.

Mice positive for antibodies to RT-P and nuclear components demonstrated presence of immunoglobulin deposits in the glomeruli. The deposits were of IgA and IgG subclasses and had a pattern often seen in the IgA nephropathy (**http://library.med.utah.edu/WebPath/RENAHTML/RENALIDX.html#10**). The glomerular immunoglobulin staining did not co-localize with the staining by anti-collagen IV antibodies (Fig. 5). The latter points that the reactivity was not specific to the immune complexes/autoantigens planted on the glomerular basement membrane (GBM) (Berden et al., 2002; Schmiedeke et al., 1989), or GBM components (as collagen, laminin, fibronectin). Instead, it was specific either to mesangial structures (Kalaaji et al., 2007), or to autoantigens that crossed GBM and get planted in the subepithelial space (Nangaku and Couser, 2005). This is supported by the similarity of immune staining with the pattern seen in kidneys of naïve mice incubated with sera positive for anti-RT-P and anti-DNA antibodies, and by the correlation of the degree of immunoglobulin deposition with the level of autoantibodies to histones. Immune complexes between autoantibodies to DNA and histones and their glomerular targets can be formed *in situ* requesting only the presence of the histone component (Morioka et al., 1996) (Nangaku and Couser, 2005). Concordantly, histones **alone** were immunostained as granular deposits in the capillaries and mesangial areas of the renal biopsies of mice with glomerulonephritis (Vogt et al., 1990) and of patients with SLE and diffuse proliferative glomerulonephritis (Stockl et al., 1994; van Bruggen et al., 1997). Altogether, this positions histones as targets for the immunoglobulin deposition in kidneys of RT-P/DNA-immunized mice with possible *in situ* formation of the complexes (not deposition of circulating immune complexes, as the latter where not detected).

The majority of mice receiving RT-P/DNA developed antibodies against reverse transcriptase (RT). However, the levels of anti-RT-P and anti-RT were not correlated, and RT-P did not inhibit antibody binding to RT. These anti-RT may have been directed against conformational

epitope(s) of the RT-P/DNA complex represented by RT co-purified in the complex with nucleic acids as was earlier indicated (Isaguliants et al., 2004). The latter is supported by the correlation of the levels of antibodies to RT and DNA. Intriguing was the detection of antibodies against RT in mice that did not receive RT-P (Table 2). Mouse chromosomes carry proviral DNA of numerous endogenous retroviruses (ERVs), many expressed as retroviral antigens (Weiss, 2006), ERV-specific antibodies constituting a prominent subset of mouse B cell repertoire (Rousseau et al., 1989). Phylogenetic analysis of ERVs and retroelements shows their high similarity to the retroviruses of today including HIV-1 specifically what concerns RT (de Parseval and Heidmann, 2005; Xiong and Eickbush, 1990). The immune stimulation and polyclonal B-cell activation enhance the antibody response against exogenous and endogenous antigens including ERV (Bos et al., 1988; Granholm and Cavallo, 1992; Hang et al., 1983; Ishigatsubo et al., 1988; Montes et al., 2007; Perl, 2003). Anti-RT response could, thus, have been induced by the polyclonal B-cell activation, with the components of immunization mixture including the adjuvant, triggering the expansion of pre-existing antibody response against the endogenous retroviral RT. Consistently, no anti-RT antibodies were induced in mock-immunized, or naïve animals. The expansion or *de novo* induction of antibodies recognizing protein RT did not lead to break of tolerance to nuclear components *per se*, as no correlation was observed between the levels of anti-RT (not recognizing RT-P) and autoantibodies in any of the control groups.

The development of autoimmune reactivities to nuclear components eventually leads to the development of autoimmune-associated disease (Ravirajan et al., 2001). Clinical observations made in the experimental systems (Chen et al., 1994; Gormly et al., 1981; Luca et al., 1995) coincide with observations made in mice immunized with RT-P/DNA complex. All manifestations, as hair loss, splenomegaly, spleen and liver fibrosis, and proteinuria, correlated with the presence and levels of autoantibodies to DNA. Further on, platelets of these mice were

in small numbers and small in size, correlating with levels of ANA. Such alterations reflecting lowered platelet activity have been described both in inflammatory and in autoimmune diseases (Ault, 2001; Park et al., 2002; Kapsoritakis et al., 2001; Mimidis et al., 2004). In the latter, the decrease in platelet size was attributed to their immune-mediated fragmentation (Ault, 2001; Mimidis et al., 2004). Altogether, this allows to interpret this clinical data as manifestations of systemic autoimmune disease. RT-P alone induced glomerular immunoglobulin deposition equal to that by the peptide/DNA complex (Fig. 4C, Fig. 6), as well as considerable proteinuria (Fig. 7). This supports the enhanced autoimmunogenicity of the DNA-binding motifs. Such peptide can bind to endogenous DNA *in situ*, with the latter serving as an autoadjuvant. This scenario was described earlier: HBcAg expressed in the tissue of the gene injected mice formed core particles incorporating host RNA that facilitated the immune response (Riedl, 2002).

We have thus shown that immunization with the canonical DNA-binding domain of the type helix-turn-helix taken from the viral polymerase can break the tolerance to DNA, with specific anti-domain antibodies being a prerequisite for the consequent development of autoimmunity. Autoimmune response to DNA spreads further to heterochromatin, histones and nuclear structures in kidneys. Such autoantibody response is associated with multiple pathological manifestations. The experiments cast new light on the mechanism of the DNA-binding proteins in the induction of autoimmunity.

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FIGURE CAPTIONS

Fig. 1 The highest titers of antibodies against the recombinant HIV-1 reverse transcriptase (RT) and its DNA-binding domain (aa 254-290; RT-P) (A); native human DNA (nDNA) and single-stranded DNA (ssDNA) (B) raised in BALB/c mice immunized with RT-P complexed to nDNA, RT-P alone, or nDNA alone, as compared to mock-immunized, or adjuvant-immunized mice.

Fig. 2 Autoantibody response against native human DNA (A) and single-stranded DNA (B) in mice positive for antibodies against HIV RT (anti-RT pos/anti-RTP neg); the DNA-binding domain of HIV-1 RT (RT-P) (anti-RTP pos/anti-RT neg); both RT and RT-P (anti-RT double pos); or negative for both antibody types (anti-RT double neg).

Fig. 3 The highest titers of autoantibodies against soluble fractions of mouse kidney proteins raised in BALB/c mice immunized with RT-P complexed to nDNA, RT-P alone, or nDNA alone, as compared to mock-immunized, or adjuvant-immunized mice. For frequency of autoantibodies against nuclear, cytoplasmic and membrane kidney protein fractions, see Table 2.

Fig. 4 Visualization of immunoglobulin deposits in mouse kidneys stained with FITC-labeled total anti-mouse Ig. Mice immunized with complex of RT-P and DNA with the Freund´s adjuvant RT-P/DNA/CFA #30(A), #27 (B); RT-P with CFA #26 (C); DNA with CFA #38 (D); primed with the complete and boosted with incomplete Freund´s adjuvant #37 (E); naïve mouse #60 (F). Magnification x20 (B, D-F) and x40 (A, C).

Fig. 5 Localization of immunoglobulin deposits in mouse glomeruli. Frozen fixed kidney sections of the BALB/c mice immunized with RT-P in complex with the fragmented double-stranded DNA in the presence of the Freund adjuvant (RT-P/DNA/CFA) were stained with: rabbit anti-collagen IV and TRITC-labeled anti-rabbit IgG and FITC-labeled anti-mouse Ig.

Fluorescence for TRITC (A) and for FITC (B); overlay of A and B (C). Dewaxed kidney section of RT-P/DNA/CFA immunized mouse stained with: HRP-conjugated anti-mouse IgA and DAB (D); HRP-conjugated anti-mouse IgG and DAB (E). Dewaxed kidney section of mock-immunized mouse stained with HRP-conjugated anti-total mouse Ig and DAB (F). White arrows, glomeruli staining; yellow arrows, anti-collagen IV staining of blood vessel and tubules.

Fig. 6 Percentage of glomeruli containing mouse immunoglobulin deposits in the BALB/c mice immunized with the DNA-binding domain of HIV-1 reverse transcriptase (RT-P) in complex with DNA, as compared to mock-immunized mice and mice immunized with single components of the complex, or adjuvant alone. Percentage of Ig-containing glomeruli was calculated by dividing the mean number of glomeruli stained by anti-mouse antibodies by the mean total number of glomeruli assessed by DAPI and anti-Col IV-immunostaining (see Materials and Methods for details).

Fig. 7 Protein content in the urine of mice immunized with a peptide representing the DNA-binding domain of HIV-1 reverse transcriptase (RT-P) in complex with DNA (RT-P/DNA/CFA), as compared to mock-immunized mice and mice immunized with single components of the complex (RT-P/CFA or DNA/CFA), or adjuvant alone (CFA/IFA).

Fig. 8 Platelet counts in BALB/c mice immunized with peptide representing the DNA-binding domain of HIV-1 reverse transcriptase (RT-P) in complex with DNA (RT-P/DNA/CFA), as compared to mice immunized with RT-P/CFA, or nDNA/CFA, or adjuvant alone. Mean values for six mice per group were recorded on a haematology analyzer after the completion of immunization cycle (week 13/day 91).

Table 1

Table 1. Individual appearance, gross pathology, hematology findings and analysis of urine of mice immunized with HIV-1 RT peptide alone or complexed to DNA as compared to control mice.
Abbreviations: n, normal; decr, decreased; incr, increased; A, anisocytosis; PC, poikilocytosis; PCR, polychromasia; HJB, Howell Jolly Bodies; Plt, platelets; PU, proteinuria; n/d, not done.

Immunogen	Mouse nn	Observations	Gross pathology at week 13	Enlarged spleen[1]	Hematology findings by blood smears and hemascreen										Protein-uria[8]
					WBC[2]	Ly[3]	Mo[4]	N segs[5]	RBC[6]	A	PC	PCR	HJB	Plt[7]	
RT-p/DNA/CFA	27	Inactive; hair loss	Ascites; splenomegaly; splenic fibrosis	+++	n	decr	incr	incr	n	+	+	-	-	n	+
RT-p/DNA/CFA	28	Inactive; hair loss	Splenomegaly; splenic fibrosis; liver fibrosis & inflammation	+++	n	decr	incr	incr	decr	+	+	-	+	n	+
RT-p/DNA/CFA	29*	Died week 12		n/d	incr	decr	incr	incr	n	+	+	+	-	decr	-
RT-p/DNA/CFA	30	Inactive; hair loss	Ascites; splenomegaly; fibrotic kidney and spleen	+++	n	decr	incr	incr	decr	+	+	+	-	decr	+
RT-p/DNA/CFA	31	Inactive; hair loss	Splenomegaly	+++	n	n	n	incr	n	+	+	+	-	n	+
RT-p/DNA/CFA	32*	Died week 7		n/d	incr	decr	n	incr	n	-	-	-	-	n	+
RT-p/CFA	21	n	Enlarged spleen; liver fibrosis & inflammation	+	n	n	n	incr	n	-	-	-	-	n	-
RT-p/CFA	22	n	Enlarged spleen	+	n	decr	incr	incr	incr	-	-	-	-	n	+
RT-p/CFA	23	n	Enlarged spleen	+	incr	n	n	incr	n	-	-	-	-	n	-
RT-p/CFA	24	n	Enlarged spleen	+	n	n	n	incr	n	-	-	-	-	incr	-
RT-p/CFA	25	Weak, inactive	None	n	incr	n	n	incr	n	-	-	-	-	n	-
RT-p/CFA	26	n	Enlarged spleen	+	decr	decr	n	incr	decr	-	-	-	-	n	-
DNA/CFA	33	n	Splenomegaly; fibrotic spleen	++	n	decr	incr	incr	n	-	+	-	-	n	-
DNA/CFA	34	n	Enlarged fibrotic spleen	++	decr	decr	incr	incr	decr	+	+	-	-	n	-
DNA/CFA	35	n	None	+	decr	decr	incr	incr	decr	+	+	-	-	n	-
DNA/CFA	36	n	None	+	decr	decr	incr	incr	decr	+	+	-	-	n	-
DNA/CFA	37	n	None	+	n	decr	n	incr	n	-	-	-	-	incr	-
DNA/CFA	38	n	None	+	n	decr	n	incr	decr	+	+	-	-	n	-
CFA	39	n	Enlarged fibrotic spleen	++	decr	decr	n	incr	decr	-	-	-	-	n	-
CFA	40	n	Enlarged fibrotic spleen	+	n	decr	n	incr	n	+	-	-	-	incr	-
CFA	41	n	Enlarged fibrotic spleen	+	n	decr	n	incr	decr	-	-	-	-	incr	-
CFA	42	n	Enlarged fibrotic spleen; fibrotic liver	++	incr	n	incr	incr	decr	-	-	-	-	n	-
CFA	43	n	Enlarged fibrotic spleen	++	n	n	incr	incr	decr	-	-	-	-	n	-
CFA	44	n	Enlarged fibrotic spleen	+	n	n	incr	incr	n	-	-	-	-	n	-
control	45	n	None	n	n	n	n	n	n	-	-	-	-	incr	-
control	46	n	None	n	n	n	n	n	n	-	-	-	-	n	+
control	47	n	None	n	n	n	n	n	n	-	-	-	-	n	-
control	1	n	None	n	n	n	n	n	n	-	-	-	-	incr	-
control	2	n	None	n	n	n	n	n	n	-	-	-	-	n	-
control	3	n	None	n	n	n	n	n	n	-	-	-	-	n	-

* No gross pathology examination was possible. Blood and urine analysis data coorespond the last available samples.

[1] Spleen weights >200 mg, +; >400 mg, ++; >600 mg, +++; splenomegaly if spleen weight >500 mg.

[2] WBC, white blood cells; if < min of the control group - STDEV, decr; if > max of the control group + STDEV, incr;

[3] Ly, Lympocytes abs and %; if both < min of the control group - STDEV, decr;

[4] Mo, Monocytes abs and %; if both > max of the control group + STDEV, incr;

[5] N segs, segmented neutraphils; if >6, incr;

[6] RBC, red blood cells; if < min of the control group - STDEV, decr; if > max of the control group + STDEV, incr;

[7] Plt, thromobocytes; if > max of the control group, incr; if < min of the control group, decr;

[8] Proteinurea by combination of qualitative evaluation by Uristrip and protein quantification by Bredford; if >= 1g/l, +; if < 1 g/l, -.

Table 2

Table 2. Frequency of autoimmune reactivites in mice immunized with peptide representing the DNA-binding domain of HIV-1 reverse transcriptase complexed to DNA (RT-P/DNA), single components of the complex RT-P or DNA, or adjuvant alone, versus "mock" immunized and naive mice. Positive for antibodies, 1; negative for antibodies, 0.

Immunogen	Mouse nn	Presence of specific Ab to		Presence of autoantibodies to								Total auto-reactivities (of 8)	Average nn of auto-reactivities
		RT-P	protein RT	nDNA	ssDNA	ANA	hetero-chromatin	histones	Anti-kidney protein fraction				
									nuclear	cytoplasmic	membrane		
RT-P/DNA/CFA	27	1	0	0	0	0	0	0	0	0	1	**1**	
RT-P/DNA/CFA	28	0	1	1	1	0	1	1	1	1	1	**7**	4
RT-P/DNA/CFA	29	1	1	1	1	1	0	1	1	1	1	**7**	(4± 2)
RT-P/DNA/CFA	30	1	0	1	1	0	1	1	0	1	0	**5**	
RT-P/DNA/CFA	31	1	0	1	0	0	0	1	0	0	1	**3**	
RT-P/DNA/CFA	32	1	1	1	0	0	0	1	0	0	0	**2**	
RT-P/DNA/CFA	16'	1	1	1	0	1	1	0	0	0	0	**3**	
RT-P/DNA/CFA	17'	1	1	1	0	0	1	0	0	0	0	**2**	
RT-P/DNA/CFA	18'	1	1	1	0	1	0	0	0	0	0	**2**	
RT-P/DNA/CFA	19'	1	1	1	1	1	1	1	1	1	0	**7**	
RT-P/DNA/CFA	20'	1	1	1	1	1	1	1	1	1	0	**7**	
RT-P/DNA/CFA	21'	1	1	1	1	1	0	0	0	0	0	**3**	
RT-P/DNA/IFA	22'	1	1	1	1	0	0	0	1	0	0	**3**	
RT-P/DNA/IFA	23'	1	1	0	0	0	1	0	0	0	0	**1**	2
RT-P/DNA/IFA	24'	1	0	1	0	0	0	0	0	0	0	**1**	(2± 1)
RT-P/DNA/IFA	25'	1	1	1	0	0	0	0	0	0	0	**1**	
RT-P/DNA/IFA	26'	1	1	1	0	0	0	0	0	0	0	**1**	
RT-P/DNA/IFA	27'	1	1	1	1	1	1	0	0	0	0	**4**	
RT-P/DNA	28'	0	1	0	0	0	0	0	0	0	0	**0**	
RT-P/DNA	29'	1	0	0	0	0	1	0	0	0	0	**1**	1
RT-P/DNA	30'	0	0	0	0	1	0	0	0	0	0	**1**	(1± 1)
RT-P/DNA	31'	1	0	0	0	0	0	0	0	0	0	**0**	
RT-P/DNA	32'	0	1	1	0	0	0	0	0	0	0	**1**	
RT-P/DNA	33'	0	1	1	0	0	0	0	0	0	0	**1**	
RT-P/CFA	21	1	0	0	0	0	0	0	0	0	0	**0**	
RT-P/CFA	22	1	0	0	1	0	0	0	0	0	0	**1**	1
RT-P/CFA	23	1	0	0	1	0	0	1	0	0	1	**3**	(1± 1)
RT-P/CFA	24	1	1	1	1	0	0	0	0	0	0	**2**	
RT-P/CFA	25	1	0	0	0	0	0	0	0	0	1	**1**	
RT-P/CFA	26	1	0	0	0	0	0	0	0	0	0	**0**	
DNA/CFA	33	0	0	0	0	0	0	0	0	0	0	**0**	
DNA/CFA	34	0	0	0	0	0	0	0	0	0	0	**0**	2
DNA/CFA	35	0	1	1	0	0	0	0	0	0	0	**1**	(2± 2)
DNA/CFA	36	0	0	0	0	0	0	0	0	1	1	**2**	
DNA/CFA	37	0	0	1	0	0	1	1	0	0	1	**4**	
DNA/CFA	38	0	0	1	0	0	0	1	0	0	1	**3**	
DNA	6'	0	0	0	0	0	0	0	0	0	0	**0**	
DNA	7'	0	1	0	0	0	0	0	0	0	0	**0**	0
DNA	8'	0	1	1	0	0	0	0	0	0	0	**1**	(0± 0)
DNA	9'	0	0	0	0	0	0	0	0	0	0	**0**	
DNA	10'	0	1	0	0	0	0	0	0	0	0	**0**	
CFA/IFA	39	1	1	0	0	0	0	0	0	0	0	**0**	
CFA/IFA	40	0	0	0	0	0	0	0	0	0	0	**0**	1
CFA/IFA	41	0	0	0	0	0	0	0	0	0	0	**0**	(1± 1)
CFA/IFA	42	0	0	0	1	0	0	0	0	0	0	**1**	
CFA/IFA	43	0	0	0	0	0	0	0	0	0	0	**0**	
CFA/IFA	44	0	0	1	1	0	0	0	0	0	0	**2**	
CFA/IFA	34'	0	0	0	0	0	0	0	0	0	0	**0**	
CFA/IFA	35'	0	1	1	1	1	0	0	0	0	1	**4**	
CFA/IFA	36'	0	1	1	0	1	0	0	0	0	0	**2**	
CFA/IFA	37'	0	1	0	0	0	0	0	0	0	0	**0**	
CFA/IFA	38'	0	0	0	0	0	0	0	0	0	0	**0**	
CFA/IFA	39'	0	1	0	0	0	0	0	0	0	1	**1**	
IFA/IFA	40'	0	1	0	0	0	0	0	0	0	0	**0**	
IFA/IFA	41'	0	0	0	0	0	0	0	0	0	0	**0**	0
IFA/IFA	42'	0	1	0	0	0	0	0	0	0	0	**0**	
IFA/IFA	43'	0	0	0	0	0	0	0	0	0	0	**0**	
IFA/IFA	44'	0	0	0	0	0	0	0	0	0	0	**0**	
IFA/IFA	45'	0	0	0	0	0	0	0	0	0	0	**0**	
mock	45	0	0	0	0	0	0	0	0	0	0	**0**	
mock	46	0	0	0	0	0	0	0	0	0	0	**0**	0
mock	47	0	0	0	0	0	0	0	0	0	0	**0**	
mock	46'	0	0	0	0	0	0	0	0	0	0	**0**	
mock	47'	0	0	0	0	0	0	0	0	0	0	**0**	
mock	48'	0	0	0	0	0	0	0	0	0	0	**0**	
naive	49'	0	1	0	0	0	0	0	0	0	0	**0**	
naive	50'	0	0	0	0	0	0	0	0	0	0	**0**	0
naive	51'	0	0	0	0	0	0	0	0	0	0	**0**	
naive	53'	0	0	0	0	0	0	0	0	0	0	**0**	
naive	54'	0	0	0	0	0	0	0	0	0	0	**0**	

Figure 1

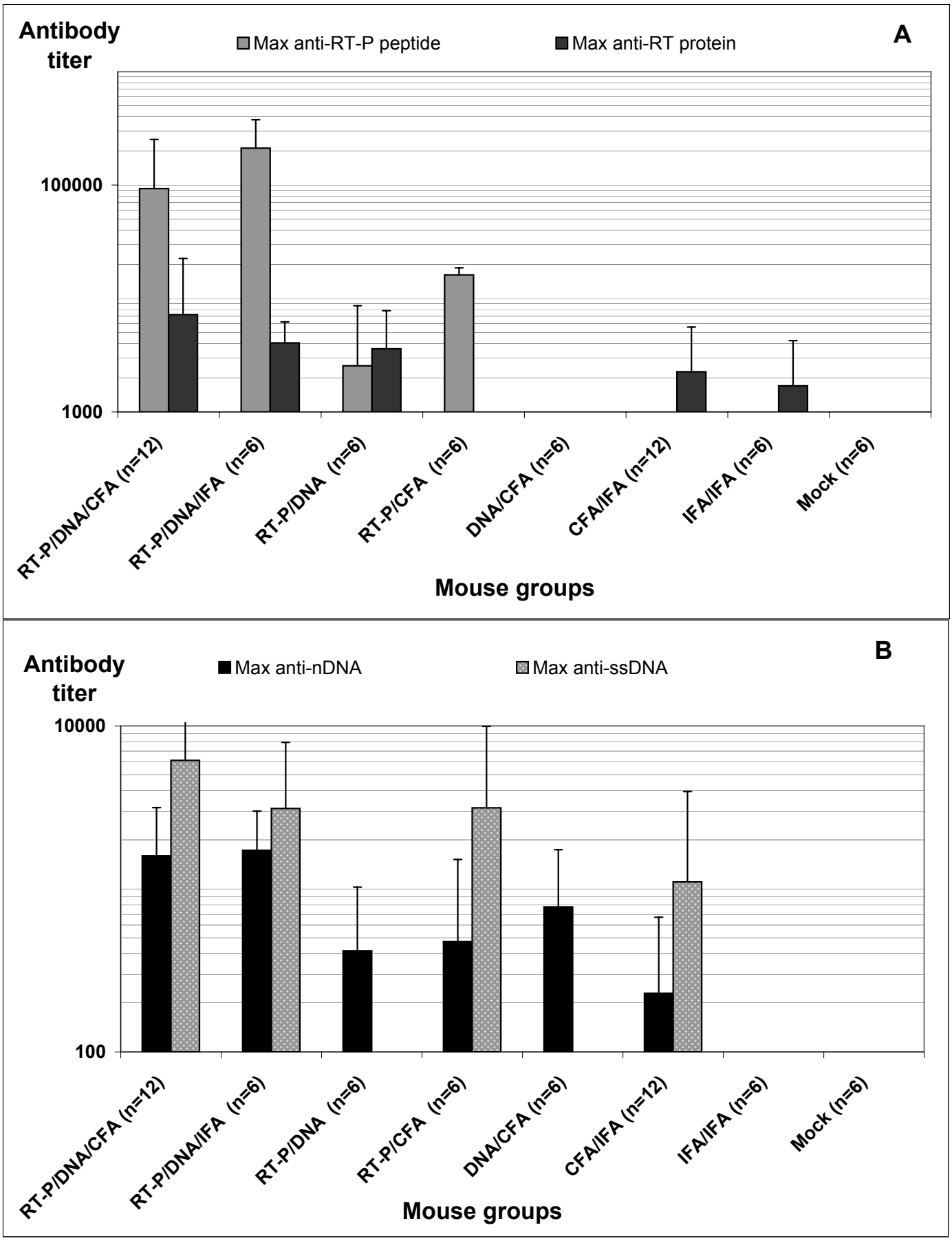
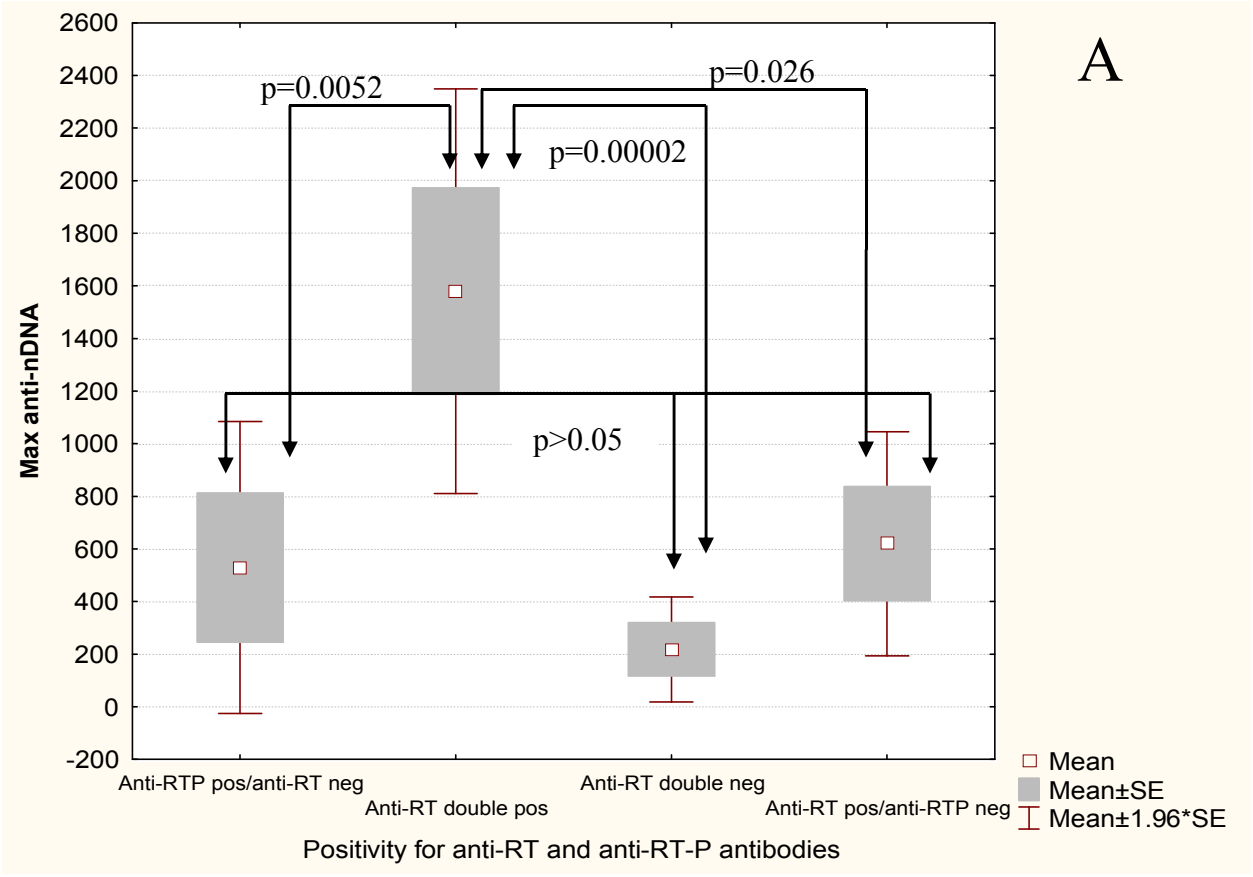

A
Antibody titer
Max anti-RT-P peptide
Max anti-RT protein
100000
1000
RT-P/DNA/CFA (n=12)
RT-P/DNA/IFA (n=6)
RT-P/DNA (n=6)
RT-P/CFA (n=6)
DNA/CFA (n=6)
CFA/IFA (n=12)
IFA/IFA (n=6)
Mock (n=6)
Mouse groups
B
Antibody titer
Max anti-nDNA
Max anti-ssDNA
10000
100
RT-P/DNA/CFA (n=12)
RT-P/DNA/IFA (n=6)
RT-P/DNA (n=6)
RT-P/CFA (n=6)
DNA/CFA (n=6)
CFA/IFA (n=12)
IFA/IFA (n=6)
Mock (n=6)
Mouse groups

Figure 2


A
Max anti-nDNA
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
-200
p=0.0052
p=0.026
p=0.00002
p>0.05
Anti-RTP pos/anti-RT neg
Anti-RT double pos
Anti-RT double neg
Anti-RT pos/anti-RTP neg
Mean
Mean±SE
Mean±1.96*SE
Positivity for anti-RT and anti-RT-P antibodies

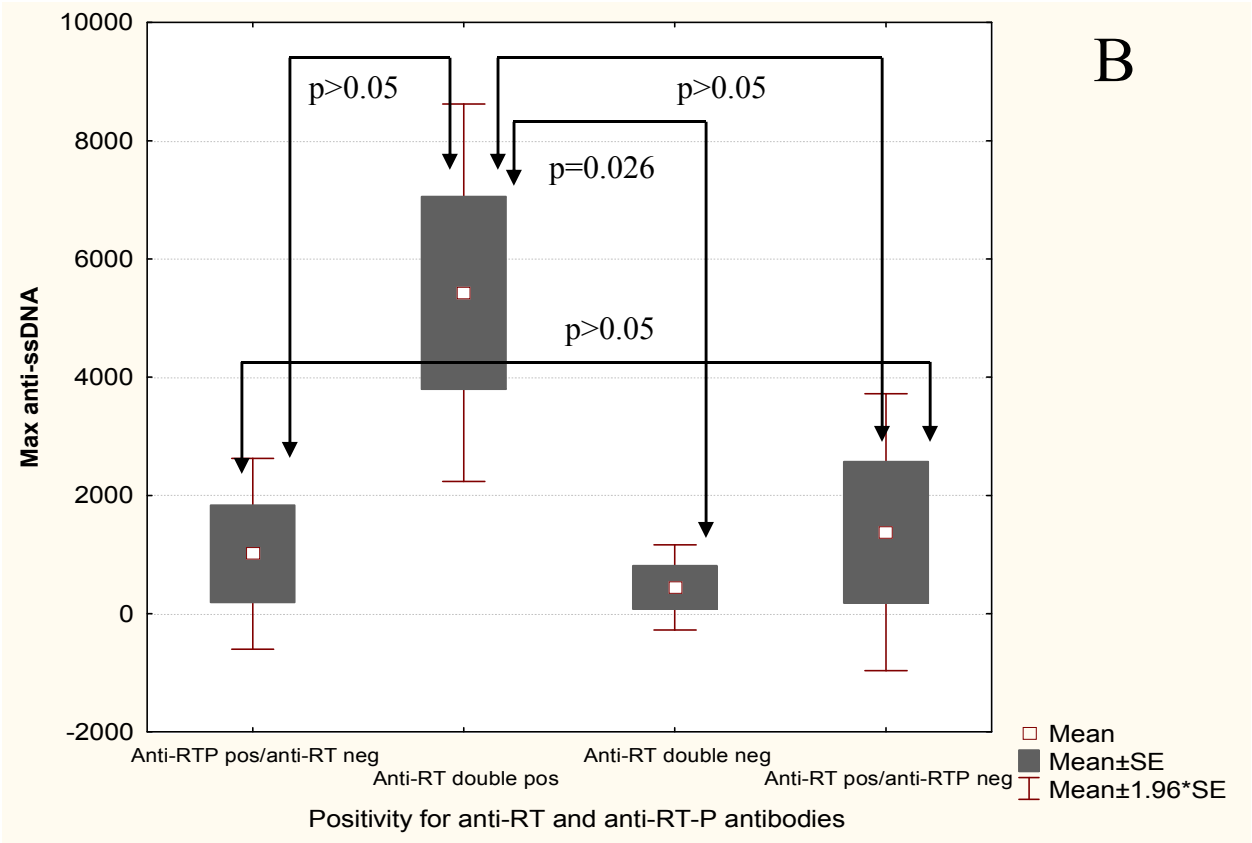

B
Max anti-ssDNA
10000
8000
6000
4000
2000
0
-2000
p>0.05
p>0.05
p=0.026
p>0.05
Anti-RTP pos/anti-RT neg
Anti-RT double pos
Anti-RT double neg
Anti-RT pos/anti-RTP neg
Mean
Mean±SE
Mean±1.96*SE
Positivity for anti-RT and anti-RT-P antibodies

Figure 3

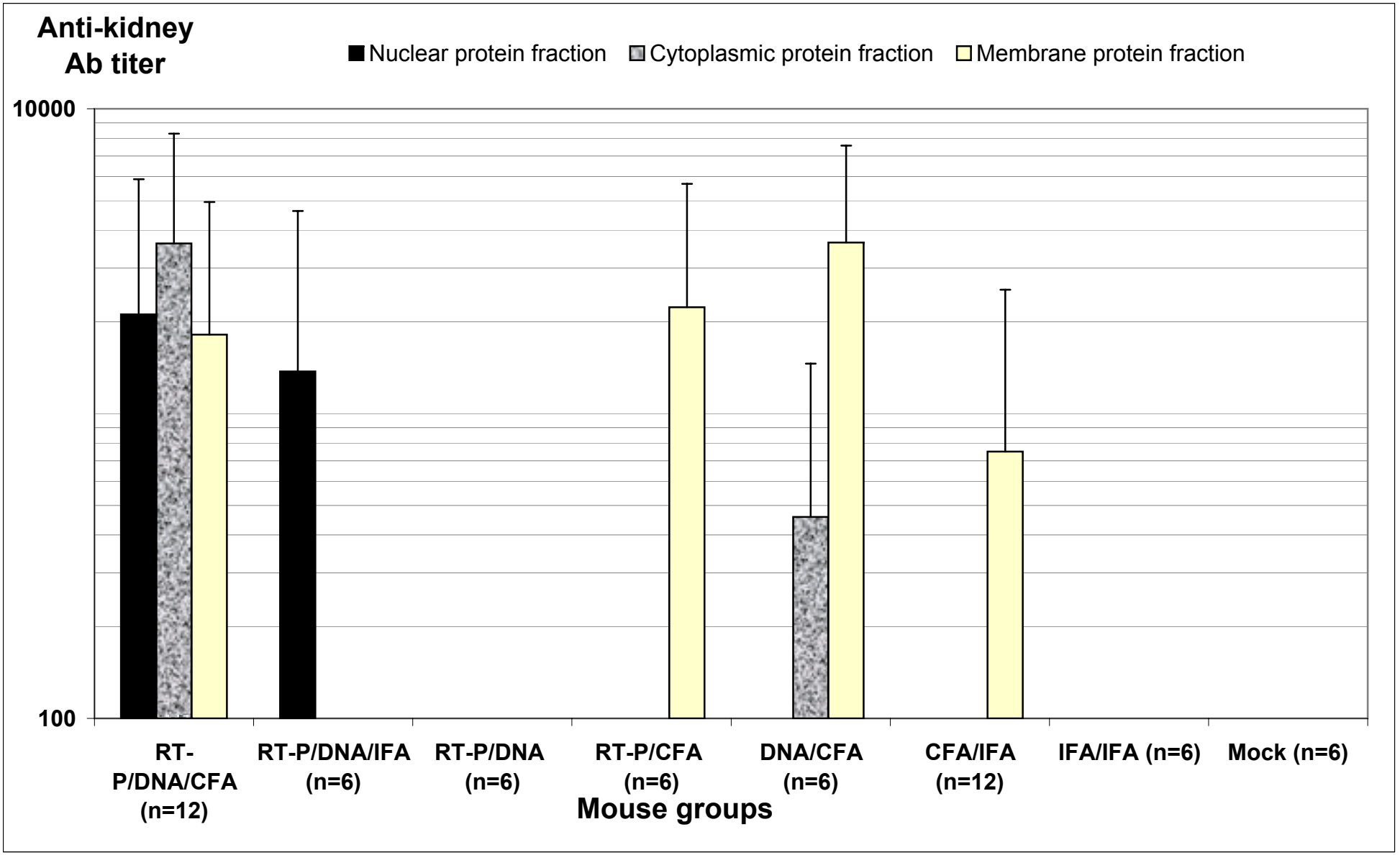

Anti-kidney Ab titer
Nuclear protein fraction
Cytoplasmic protein fraction
Membrane protein fraction
10000
100
RT-P/DNA/CFA (n=12)
RT-P/DNA/IFA (n=6)
RT-P/DNA (n=6)
RT-P/CFA (n=6)
DNA/CFA (n=6)
CFA/IFA (n=12)
IFA/IFA (n=6)
Mock (n=6)
Mouse groups

Figure 4

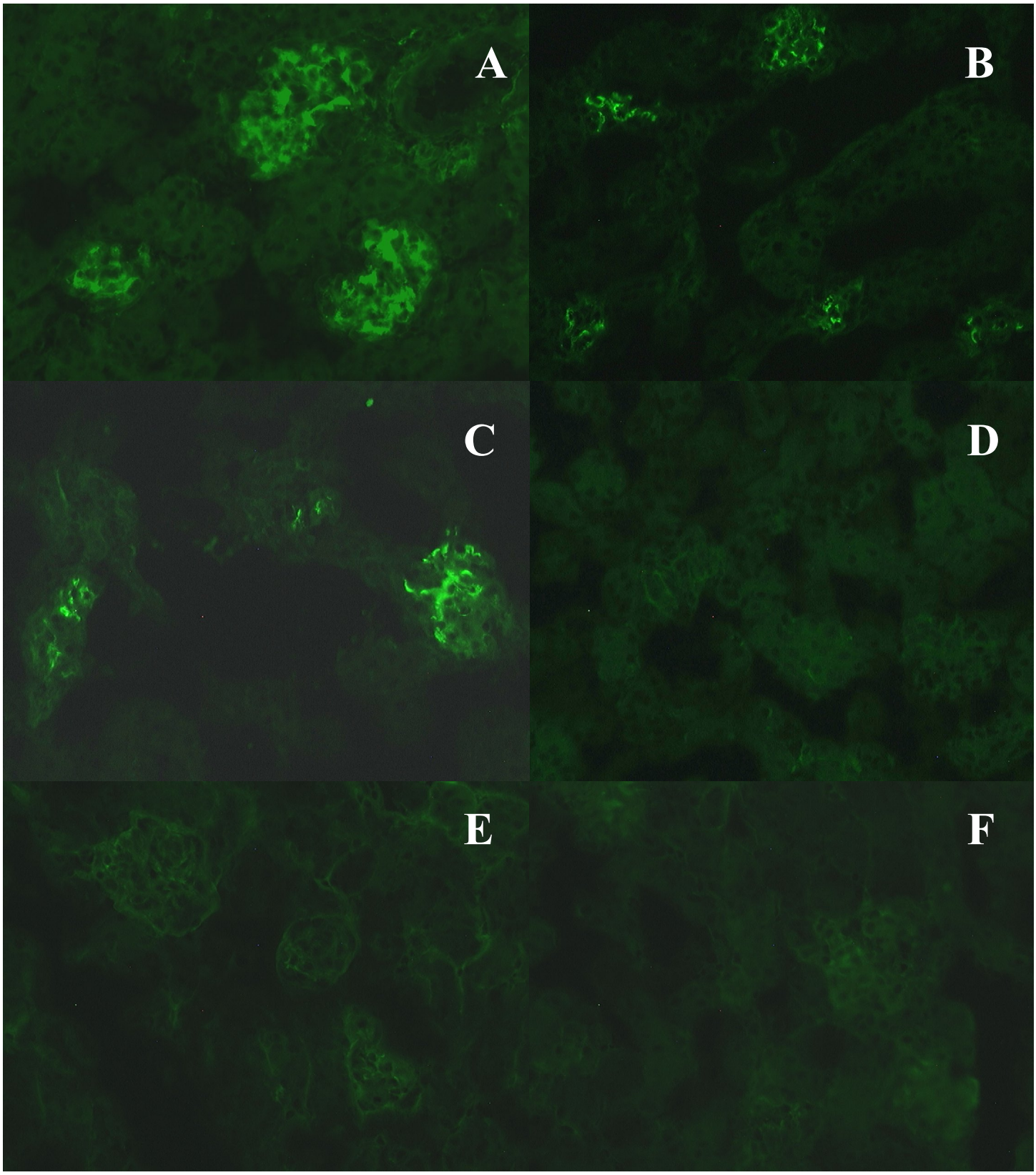


Figure 5

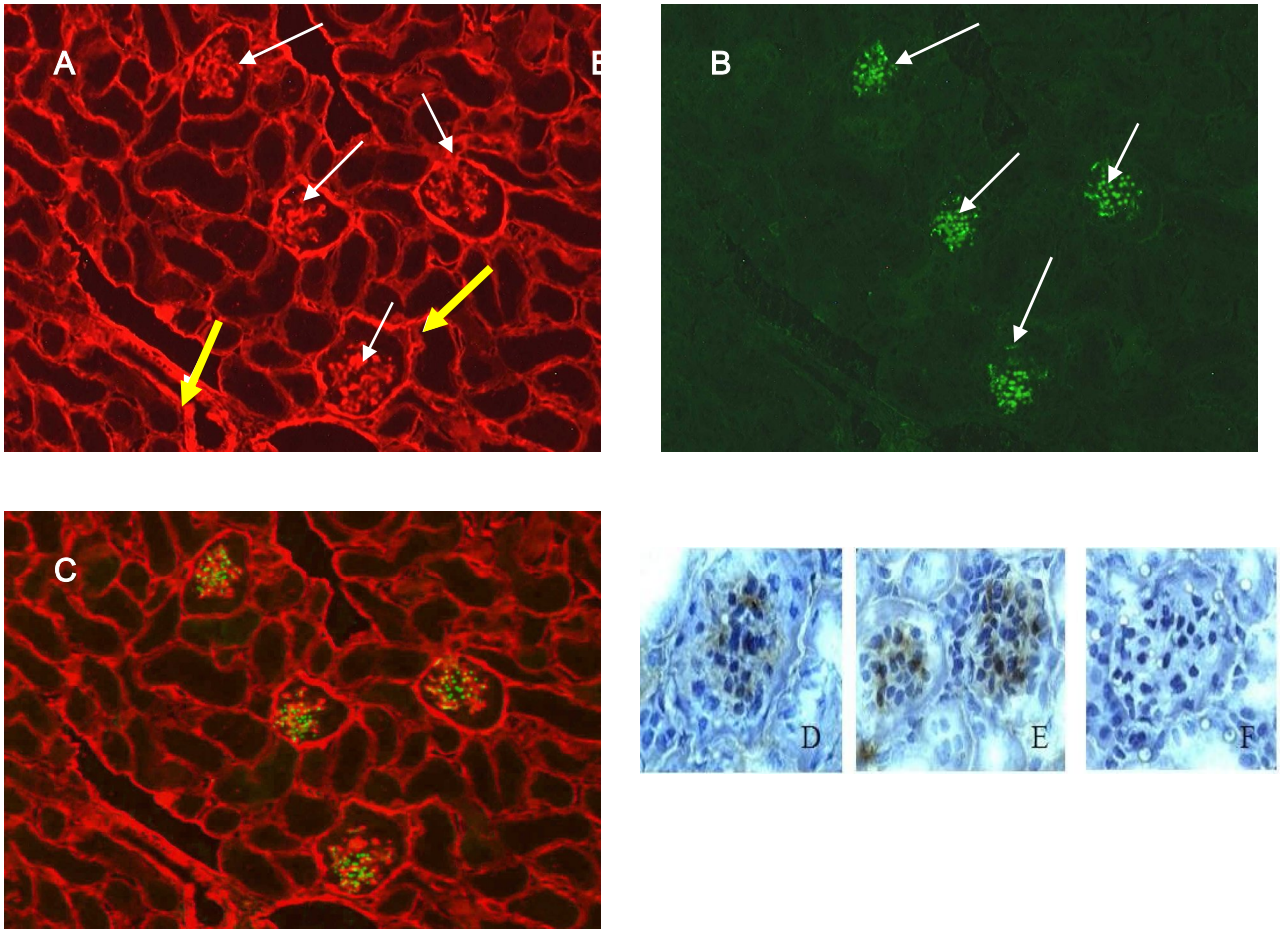


Figure 6

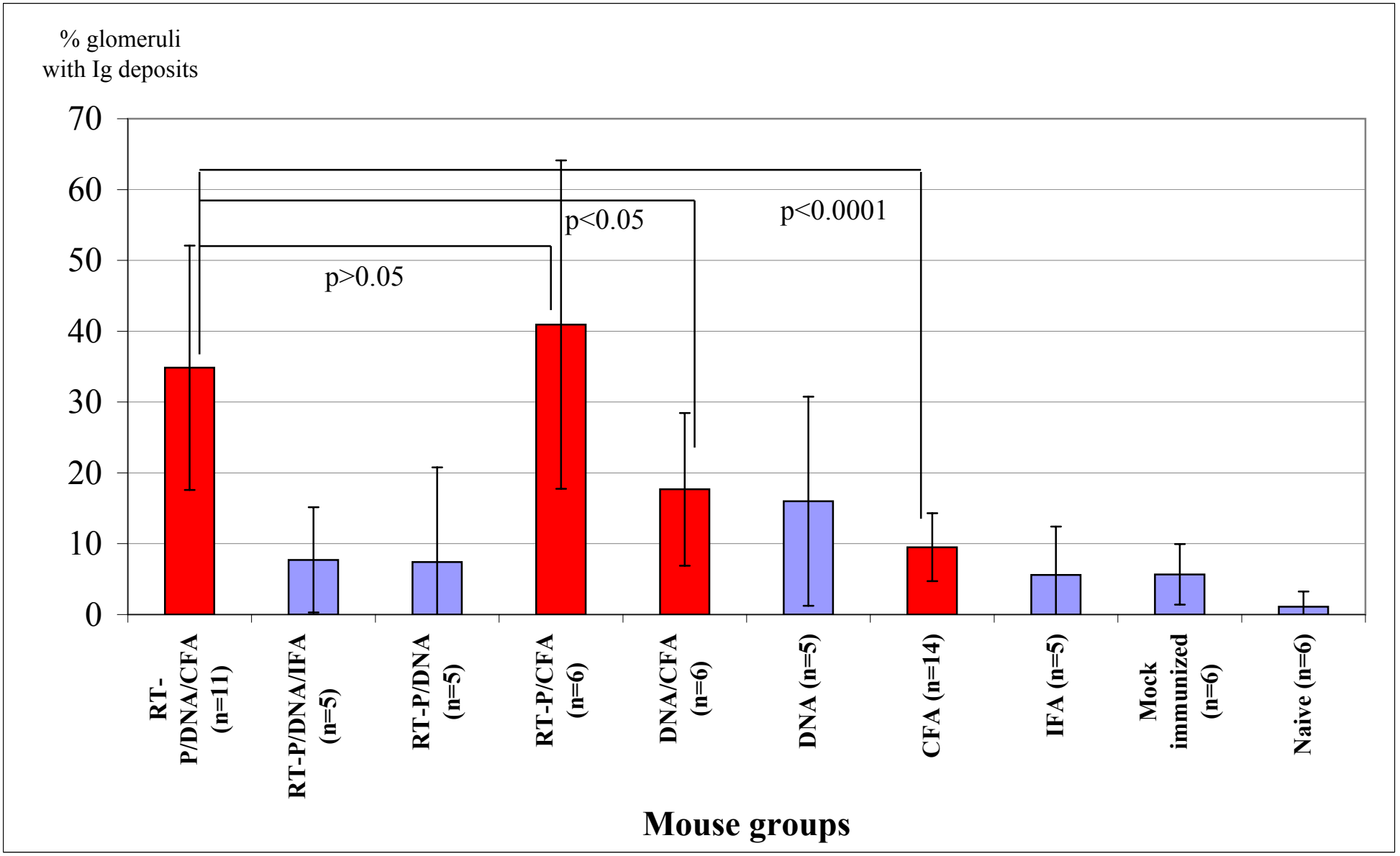

% glomeruli with Ig deposits
70
60
50
40
30
20
10
0
p>0.05
p<0.05
p<0.0001
RT-P/DNA/CFA (n=11)
RT-P/DNA/IFA (n=5)
RT-P/DNA (n=5)
RT-P/CFA (n=6)
DNA/CFA (n=6)
DNA (n=5)
CFA (n=14)
IFA (n=5)
Mock immunized (n=6)
Naive (n=6)
Mouse groups

Figure 7

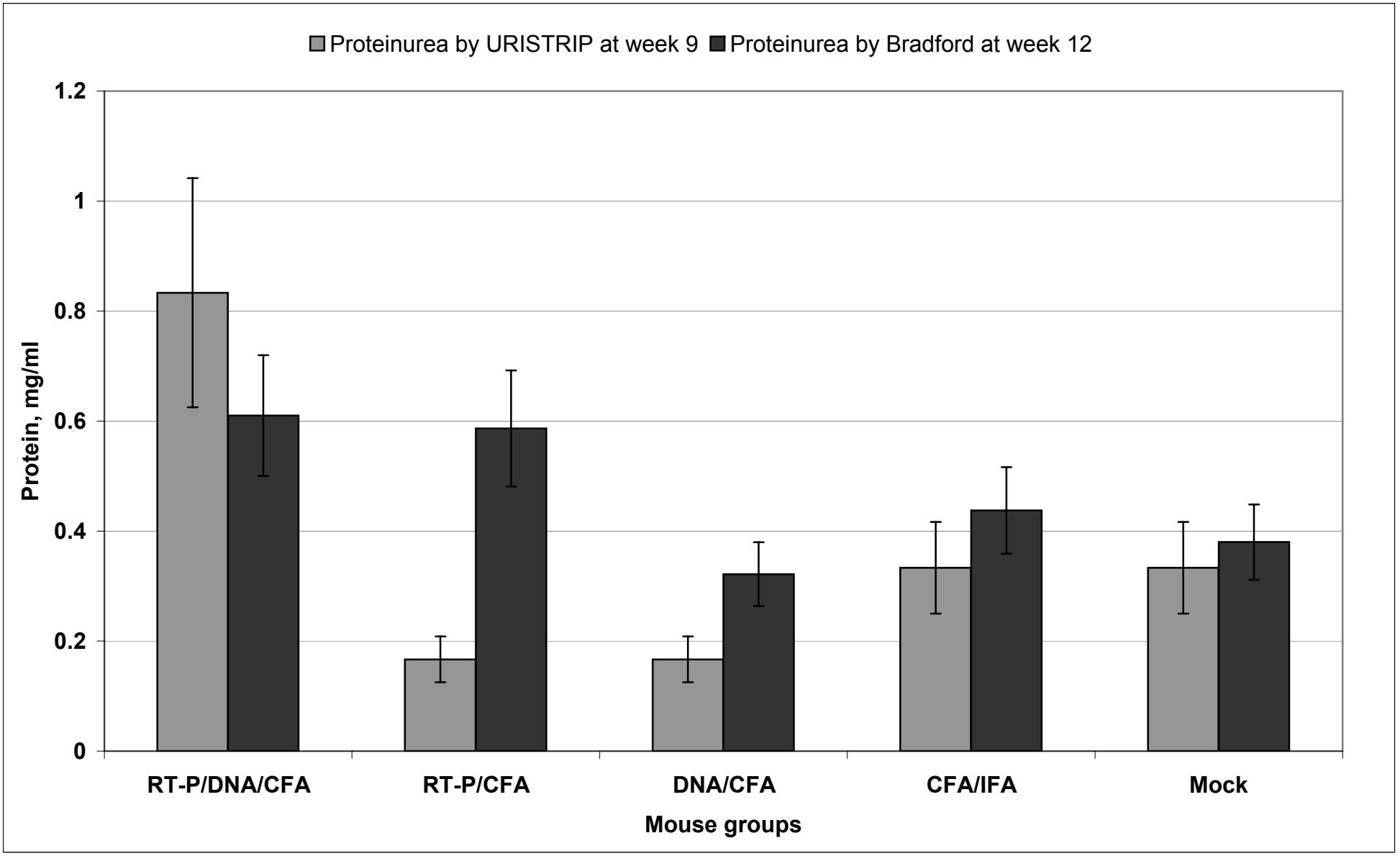
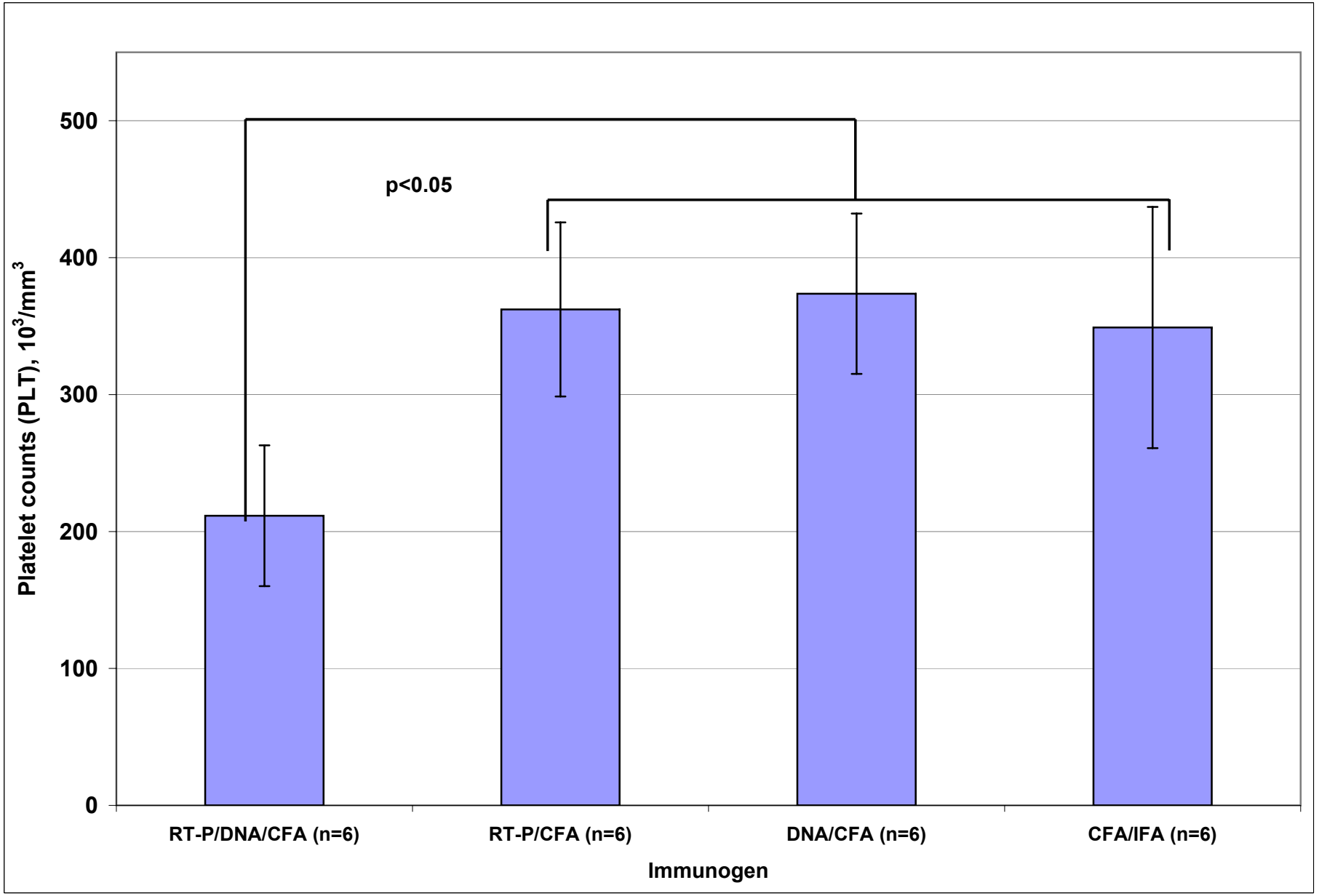

Proteinurea by URISTRIP at week 9
Proteinurea by Bradford at week 12
1.2
1
0.8
0.6
0.4
0.2
0
Protein, mg/ml
RT-P/DNA/CFA
RT-P/CFA
DNA/CFA
CFA/IFA
Mock
Mouse groups

Figure 8


p<0.05
500
400
300
200
100
0
Platelet counts (PLT), 10^3/mm^3
RT-P/DNA/CFA (n=6)
RT-P/CFA (n=6)
DNA/CFA (n=6)
CFA/IFA (n=6)
Immunogen

ABSTRACT

Nonimmunogenic character of native DNA, and its high immunogenicity when presented in complex with the DNA-binding proteins indicate that the latter might contain molecular triggers of anti-DNA response. To find if this is the case, we have evaluated the autoimmunogenic potential of the main DNA-binding domain of HIV-1 reverse transcriptase that belongs to the canonical helix-loop-helix type. BALB/c mice were immunized with a peptide representing the domain, alone or in complex with the fragmented human DNA in the presence of an adjuvant. Mice were assessed for specific antibodies, autoantibodies against a panel of self-antigens; glomerular immunoglobulin deposition; and for the signs of autoimmune disease, such as proteinuria, and changes in the blood compoments. Immunization with the adjuvanted peptide-DNA complex induced autoantibodies against double-stranded DNA, histones, heterochromatin, and kidney proteins; glomerular IgG and IgA deposition; proteinuria; thrombocytopenia, and anemia. Altogether, this identifies the helix-loop-helix DNA-binding domain as one of the molecular triggers of autoimmunity to DNA and DNA-associated proteins. The experiments cast new light on the role of the DNA-binding retroviral proteins in the induction of autoimmunity, and on the origins of autoimmune complications in the microbial infections in general. It also implies that choosing the DNA-binding proteins as vaccine candidates should be done with precaution.

Key words: DNA-binding domain; reverse transcriptase; anti-DNA autoantibody; heterochromatin; histones; proteinuria; nephropathy; thrombocytopenia.