

ORIGINAL ARTICLE

The context of tetanus toxoid application influences the outcome of antigen-specific and self-directed humoral immune response

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ABSTRACT

Results are presented concerning our attempts to create a suitable model system for studying the connection between microbial antigen (micAg), autoimmunity and autoimmune disease on the basis of hyper-immunization and application of micAg in different contexts. Our research was focused on tetanus toxoid (TTd) as a model micAg. Non-pretreated and complete Freund's adjuvant pretreated BALB/c mice were immunized with high doses of TTd mixed with glycerol or aluminum hydroxide as adjuvants. The main aims of the experiments were to evaluate the properties of induced humoral immune responses, evaluate the pathological potential of induced immune responses and determine possible correlations between the properties of a humoral immune response and its pathological potential. The production of TTd-specific and self-reactive β_2 -glycoprotein I (β_2 -GP I)-specific antibodies (Abs) was detected in all groups but with specific, context-related properties. Analysis of pregnancy-related pathology (anti- β_2 -GP I Abs-associated) showed differences in the pathological potential of the induced immune response. It was demonstrated that severity of pathology is positively correlated to the abundance of IgG that recognizes β_2 -GP I adsorbed onto phosphatidylserine, and to IgG affinity. Furthermore, it was demonstrated that molecular mimicry, which results in generation of anti- β_2 -GP I Abs upon TTd immunization, is necessary but not sufficient for the development of pregnancy-related pathology.

Key words adjuvant, anti- β_2 -glycoprotein I antibody, tetanus toxoid.

Various data that connect exposure to micAg, during infections (1–4) and vaccinations (5–8), to autoAb production have been published in recent years. Among the possible mechanisms that contribute to autoAb generation, the most cited mechanisms are molecular mimicry (9, 10) and polyclonal cell activation (11, 12). Available data show that long-term stimulation by micAg is accompanied by augmentation of self-reactivity, but not by obvious induc-

tion of autoimmune diseases (13). Hence, we have tried to create a model system for studying the connection between micAg, autoimmunity and autoimmune disease on the basis of hyper-immunization and application of micAg in different contexts.

With the intention of creating a suitable model system, we focused our research on TTd, a chemical derivative of tetanus toxin, as a model antigen. As a single protein

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List of Abbreviations: Ab, antibody; Ag, antigen; Al, aluminium hydroxid; APS, anti-phospholipid syndrome; autoAb, autoantibody; β_2 -GP I, β_2 -glycoprotein I; CFA, complete Freund's adjuvant; CFA//, CFA-pretreated; glyc, glycerol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IL, interleukin; KSCN, potassium thiocyanate; micAg, microbial antigen; nc, normal age-matched control; no//, non-pretreated; NoF, number of full-term fetuses; NoFR, number of fetal resorptions; OPD, o-phenylenediamine; PI, percentage of inhibition; PS, phosphatidylserine; SCN⁻, thiocyanate ion; [SCN⁻], SCN⁻ concentration; [SCN⁻]_{50%}, [SCN⁻] that induced a 50% reduction of initial absorbance; Th, T helper; TLR, Toll-like receptor; TTd, tetanus toxoid; TTd/Al, TTd mixed with 2% aluminum hydroxide; TTd/glyc, TTd mixed with 2.5 M glycerol.

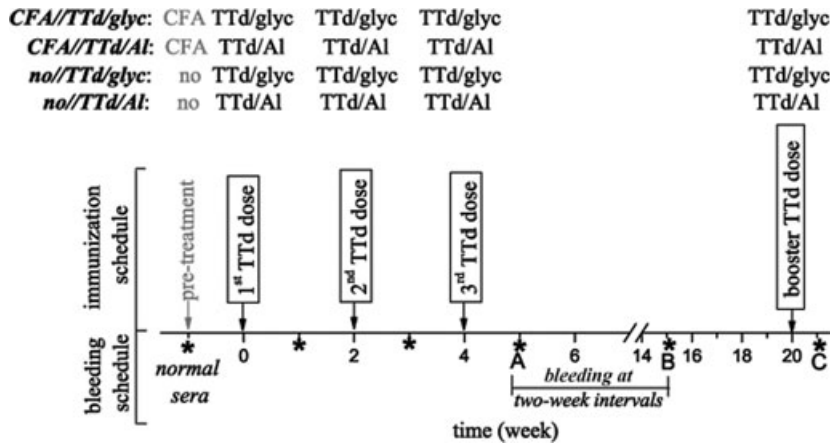


Fig. 1. Immunization and bleeding schedules.

molecule, TTd is less complex and more easily characterized than bacterial cell or viruses, but at the same time fulfills the criteria for induction of autoimmunity. Tetanus toxoid and several serum proteins have similar structure at either the three-dimensional level (IL-1 α , IL-1 β) (14) or the level of short peptide sequence homology (β_2 -GP I) (15) and laminin (16)). This structural similarity favors the role of TTd in autoimmunity, with molecular mimicry as the mechanism. These facts serve as the basis for tracking anti- β_2 -GP I and anti-laminin Abs as markers of a self-reactive immune response following TTd immunization.

In the murine immune system, anti-laminin Abs have obvious pathological attributes (17), whereas anti- β_2 -GP I Abs can exert both beneficial (playing a homeostatic role) and deleterious effects, depending on their concentration, isotopic characteristics, affinity and fine epitope specificity (18). The pathological anti- β_2 -GP I Abs represent a molecular substrate of the APS, a systemic autoimmune disorder characterized by arterial and venous thrombosis and/or pregnancy-related pathology (19).

To induce maximal immune system activation, we immunized BALB/c mice with high concentrations of TTd (20). Aluminum hydroxide or glycerol were used as adjuvants to promote the humoral (21–23) and cellular (24) arms of the immune response, respectively. TTd immunizations were performed on non-pretreated or pretreated mice (single dose of CFA injected subcutaneously one week before the start of immunization). By combining specific TTd immunization (with glycerol or aluminum hydroxide as adjuvants) with CFA pretreatment, we were able to hyper-stimulate both arms of the immune system simultaneously (25).

The main aims of the experiments were to 1) evaluate properties of humoral immune responses induced by TTd as applied in different contexts; 2) evaluate the pathological potential of the induced immune responses and 3)

determine possible correlations between the properties of the humoral immune response and its pathological potential. Obtained results clearly demonstrated that the properties of induced immune responses were context-dependent, thus providing a basis for the establishment of a new animal model to examine the underlying mechanisms of the association between micAg, autoimmunity and autoimmune disease.

MATERIALS AND METHODS

Immunization and bleeding schedules

Ten-week-old BALB/c female mice were used in the experiments. Immunization and bleeding schedules are presented in Figure 1. Briefly, pretreatments (single dose of CFA injected subcutaneously) were performed one week before the first TTd application. Non-pretreated and CFA-pretreated mice were immunized with high doses of TTd (Institute, Torlak, Belgrade) mixed with either 2.5 M glycerol (TTd/glyc) or 2% aluminum hydroxide (TTd/Al) as adjuvants (three times at 2-week intervals, 500 μ g/ml TTd per dose, 200 μ l/mouse, subcutaneously). Sixteen weeks after the third dose, one additional dose of TTd was administered with an appropriate adjuvant (the booster dose). TTd used for immunization had passed the tests of specific and reversed toxicity according to European Pharmacopoeia requirements. According to the pretreatments and immunization protocols applied, the whole experimental mice population was divided into four groups (15 mice per group). In addition, a group (10 mice) of non-treated BALB/c mice was used as normal age-matched controls.

Samples of blood sera were collected by bleeding from the retro-orbital plexus before any intervention (normal sera), and subsequently at 2-week intervals during a period of more than 3 months and 1 week after the booster

TTd dose application. The collected sera were complement depleted, aliquoted and stored at -20°C until used for analyses. For serological assays, individual sera or sera pools (mixtures containing the same volume of sera obtained from identically immunized mice at the same time point in relation to the start of treatment) were used as samples. The main check points were before any intervention (normal sera), 1 week after the third TTd dose (check point A), 11 weeks after the third TTd dose (check point B) and 1 week after the booster TTd dose application (check point C). Corresponding sera pools were assigned as normal, A, B and C, respectively.

All animal experimentation was conducted in accordance with the local "Guiding Principles for the Care and Use of Laboratory Animals", conformed with the provisions of the Declaration of Helsinki and was approved by the Animal Institutional Care and Use Committee at the Institute, Torlak, Belgrade.

Isolation of β_2 -GP I from human plasma

β_2 -GP I, which was used for serological tests, was isolated from human plasma. Briefly, 70% perchloric acid (2.5 ml) was added to human plasma (100 ml) and stirred for 15 min on ice. After centrifugation (15 min, 13 000 g, 4°C ; 3K18 Sigma Laboratory Centrifuge, Osterode, Germany), the supernatant was collected and the pH neutralized. Precipitation of β_2 -GP I was performed by addition of ammonium sulfate to 65% saturation. Following incubation (stirred for 30 min on ice) and centrifugation (15 min, 13 000 g, 4°C), the pellet was resuspended in 0.05 M NaCl/0.05 M HEPES, at pH 7, and dialyzed against the same solution over night at 4°C . Isolation of β_2 -GP I was done by ion-exchange chromatography on a Mono S column (Pharmacia Amersham, Uppsala, Sweden) connected to a high-performance liquid chromatography system (ÄKTA Purifier; Pharmacia Amersham). The bound β_2 -GP I was eluted by a linear gradient of NaCl concentration (0.05–0.5 M), checked for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%–15% polyacrylamide gel; Phast System; Pharmacia Amersham) and identified as β_2 -GP I by immune-blotting analysis, with the use of commercially available mouse anti-human β_2 -GP I IgG1 (clone 5F7; ICN Biomedicals, Aurora, IL, USA).

Indirect ELISA

Detection of sera Abs specific for TTd, β_2 -GP I or laminin was performed by indirect ELISA. In all cases the ExtrAvidin-peroxidase/OPD system (Sigma, Steinheim, Germany) was used to visualize Ag–Ab interactions. Absorbance was monitored at 492 and 692 nm ($A_{492/692}$). The cut-off value for each system was defined according

to the $A_{492/692}$ read in "negative control" wells (1% BSA; w/v in PBS as a sample) plus $3 \times \text{SD}$. Serum samples were considered positive when the $A_{492/692}$ value exceeded the cut-off value.

Detection of Abs specific for TTd and β_2 -GP I

ELISA plates (MaxiSorp; Nunc, Roskilde, Denmark) were covered (50 μl /well) with appropriate Ag – TTd (1 $\mu\text{g}/\text{ml}$ TTd in PBS) or β_2 -GP I (10 $\mu\text{g}/\text{ml}$ β_2 -GP I in PBS) by overnight adsorption at 4°C . One percent BSA (w/v) in PBS (1% BSA/PBS) was used for blocking for 2 hours at room temperature. The blocking, as well as all subsequent ELISA steps, were followed by washing with 0.05% (v/v) Tween 20 in PBS (four times, 200 μl /well). Appropriately diluted serum samples were incubated for 1 hour at room temperature. Ag-specific Ab binding was detected with the following biotin-labeled Abs (used according to the manufacturer's instructions): anti-mouse IgM (Cat. No. B-9265; Sigma, St. Louis, MO, USA), anti-mouse IgG (Cat. No. B-7401; Sigma, Steinheim, Germany), anti-mouse IgG1 (clone LO-MG1–2; ICN Biomedicals) or anti-mouse IgG2a (clone LO-MG_{2a}-3; ICN Biomedicals). An appropriate biotin-labeled Ab (50 μl /well) was incubated for 1 hour at room temperature.

Serum samples were diluted 1:800, except for detection of anti-TTd IgG (1:1000) and anti- β_2 -GP I subclass evaluation (1:500).

Detection of sera IgG specific for β_2 -GP I adsorbed onto PS

PS (Sigma, Steinheim, Germany) (10 $\mu\text{g}/\text{ml}$, 50 μl /well) was immobilized onto ELISA plates (PolySorp; Nunc) by evaporation at room temperature. Then, β_2 -GP I (10 $\mu\text{g}/\text{ml}$, 50 μl /well) was coated by overnight incubation at 4°C . The steps that followed were the same as those described for detection of anti- β_2 -GP I Abs.

Detection of sera IgG specific for mouse laminin

Mouse laminin (Sigma, St. Louis, MO, USA) (10 $\mu\text{g}/\text{ml}$, 50 μl /well) was adsorbed onto microtiter plates (MaxiSorp; Nunc) for 4 hours at 37°C . The plates were then washed (three times, 200 μl /well PBS) and blocked with 1% BSA/PBS. Samples (sera diluted 1:200 in 1% w/v BSA, 0.05% v/v Tween 20 in PBS) were added to the plates after washing (four times, 200 μl /well 0.05% v/v Tween 20 in PBS) and incubated overnight at 4°C . Bound IgG was detected after incubation with biotin-labeled anti-mouse IgG (Sigma, Steinheim, Germany).

Competitive ELISA for detection of Y7 natural idiotope concentration changes in sera of immunized mice

For the measurement of Y7 expression, two monoclonal Abs were used: biotin-labeled F(ab)₂ of the mouse

monoclonal anti-idiotopic antibody Y7 (F(ab)₂ Y7-B) and Y7+ human monoclonal Ab IgM DJ. The Y7 concentration was estimated via inhibition of F(ab)₂ Y7-B binding to IgM DJ adsorbed onto plastic. IgM DJ was bound on a microtiter plate (MaxiSorp; Nunc) (50 µl/well) at a concentration of 500 ng/ml. The F(ab)₂ Y7-B (200 ng/ml) was mixed with mice sera diluted in 1% w/v BSA in PBS solution (final dilution of 1:100), pre-incubated for 1 hour at 25°C in a water bath and further incubated with IgM DJ coated on the microplate walls. ExtrAvidin-peroxidase/OPD was used as a detection system. The PI was calculated after determination of free F(ab)₂ Y7-B from the standard curve:

$$A_{492/692} = f([F(ab)_2 Y7 - B]) \quad [1]$$

For each group, the PI values at defined time point were analyzed and corresponding PI ranges were determined. Intensity of fluctuations was evaluated according to calculated PI ranges.

Affinity determination by ELISA of IgG specific for TTd and β₂-GP I

The affinities of anti-β₂-GP I IgG and anti-TTd IgG to their Ags were estimated by the use of aqueous solutions containing different KSCN concentrations. Analysis of the influence of SCN⁻ on specific sera IgG binding was performed with ELISA-based procedures. The protocol was similar to those performed for the detection of IgG specific for TTd and β₂-GP I. The unique difference was an additional incubation of 30 min with KSCN aqueous solutions after the binding of samples to adsorbed Ag. The aqueous solutions containing increasing KSCN concentrations (0–6 M) were used for Ab desorption. The [SCN⁻]_{50%} was calculated for each sample upon linearization of the corresponding Ag–Ab dissociation profile:

$$\text{binding}(\%) = f([\text{SCN}^-]) \quad [2]$$

A preliminary experiment showed that KSCN in the concentrations used did not induce desorption of coated β₂-GP I or TTd.

Pregnancy-related pathology

Ten-week-old BALB/c mice were immunized with TTd according to the described protocols (Fig. 1) and mated after the third TTd dose. Non-treated mice (normal age-matched controls) were also mated. The date of coitus was determined by visualization of the vaginal plug (denoted as the first day of pregnancy). Mice were killed on the 14th day of pregnancy, and macroscopic examination of the embryos and uterus performed. The NoF as well as NoFR (a more than 50% reduction in fetal volume) was determined. The rate of fetal resorption was calculated for

each mouse and expressed as the percentage of the total number of fetuses:

$$\text{NoFR} \times 100 / (\text{NoF} + \text{NoFR}) \quad [3]$$

Statistical analysis

The statistical significance of immunization-induced fluctuations in a defined Ab pool was determined by a paired Student's *t*-test, using values for corresponding normal sera collected prior to any intervention as a reference. For estimation of the TTd-booster dose influence (check point C), the concentrations of specific Abs at check point B were used as a reference.

To evaluate the significance of the observed differences in sera IgG affinity toward TTd and β₂-GP I, dissociation profiles were also compared by a paired Student's *t*-test.

Severity of pregnancy-related pathology (resorption rates, number of fetuses) was evaluated by a *t*-test for independent groups. The control group of non-treated mice was used as a reference.

In all cases, a *P*-value of 0.05 was considered the limit of statistical significance.

RESULTS

Anti-TTd Abs

Concentrations of TTd-specific IgM and IgG were measured in individual mice sera; their mean concentrations at defined time points are presented in Figure 2. Analysis of *nc* group sera did not show any significant time-dependant fluctuations within these pools (data not shown).

Anti-TTd IgM

TTd-specific IgM was detected in normal sera collected before any treatment (mean $A_{492/692} \pm \text{SE} = 0.243 \pm 0.011$) (Fig. 2a). A transient fall in the anti-TTd IgM concentration was recorded in all groups during the course of immunization (Fig. 2a). During the post-immunization follow-up period, a slight increase in TTd-specific IgM was observed in all groups except CFA//TTd/Al. The booster dose application induced an increase in TTd-specific IgM concentration in both non-pretreated (*P* < 0.05) and CFA//TTd/Al groups.

Anti-TTd IgG

IgG specific for TTd was absent in sera collected prior to any treatment (mean $A_{492/692} \pm \text{SE} = 0.064 \pm 0.003$) (Fig. 2b). Immunization induced a gradual increase in the concentration of anti-TTd IgG (predominantly IgG1, data not shown), and the concentration was significantly increased in all groups after completion of the immunization protocol (check point A).

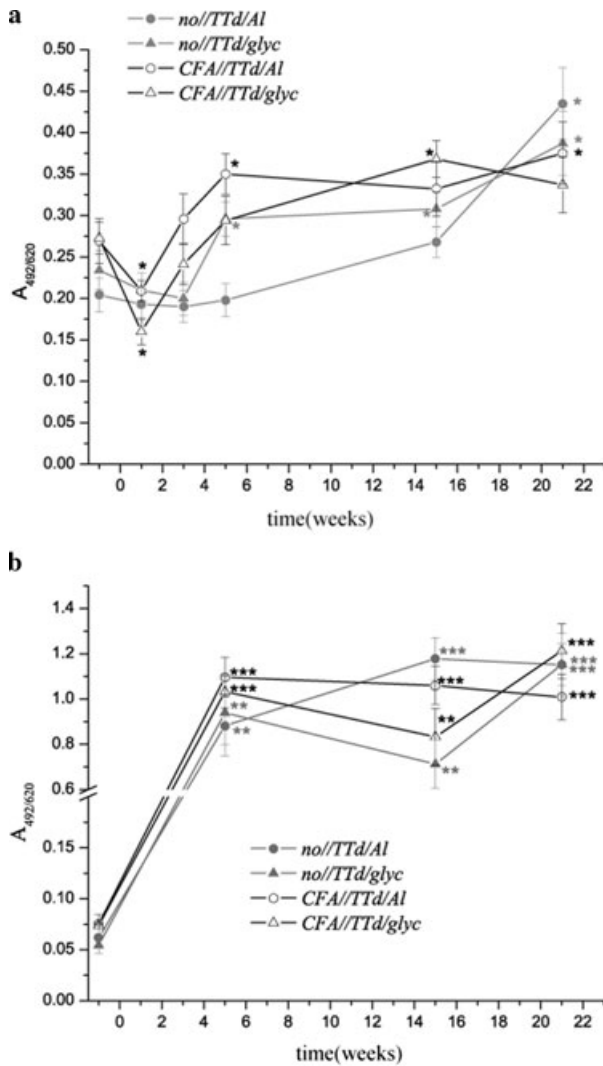


Fig. 2. Immunization-induced changes in the pool of IgM (panel a) and IgG (panel b) specific for TTd. The reactivity of Abs belonging to the same isotype towards TTd was examined simultaneously in individual sera. Results are presented as the mean $A_{492/620} \pm$ SE for each group ($n = 15$ mice/group) at defined time points. The statistical significance of immunization-induced fluctuations was determined by a paired Student's *t*-test using the $A_{492/620}$ for corresponding normal sera (-1 week) as a reference ($P < 0.05^*$, $P < 0.005^{**}$, $P < 0.0005^{***}$). Cut-off values for detection of IgM and IgG Abs specific for TTd were 0.03 and 0.05, respectively.

Despite a slight fall observed during the 11-week post-immunization follow-up period in all TTd/glyc groups (Fig. 2b), the concentration of anti-TTd IgG remained at a significantly higher concentration compared with the corresponding normal sera. In addition, the booster TTd dose induced an additional anti-TTd IgG increase in those groups ($P < 0.05$, compared with the sera taken at check point B).

In the no//TTd/Al group a sustained increase in anti-TTd IgG during the post-immunization period was observed, with a maximal level recorded 11 weeks after the last TTd dose (Fig. 2b). The concentration of anti-TTd IgG after the booster dose remained unaffected in both TTd/Al groups.

Anti-TTd IgG affinity

The dissociation profiles of TTd/anti-TTd IgG complexes in the presence of different KSCN concentrations were used to estimate affinity of sera anti-TTd IgG toward TTd. Pools of sera taken at check point A were used as samples. The linear correlation ($R > 0.98$) between $[\text{SCN}^-]$ and anti-TTd IgG binding was calculated. The average affinities of anti-TTd IgG, evaluated according to $[\text{SCN}^-]_{50\%}$, were higher in sera from TTd/Al-immunized mice ($[\text{SCN}^-]_{50\%}$ no//TTd/Al = 3.19 M; $[\text{SCN}^-]_{50\%}$ CFA//TTd/Al = 4.36 M) compared with the corresponding TTd/glyc under the same pretreatment protocol ($[\text{SCN}^-]_{50\%}$ no//TTd/glyc = 3.28 M; $[\text{SCN}^-]_{50\%}$ CFA//TTd/glyc = 3.82 M). As can be seen, IgG Abs with the highest affinity toward TTd were present in sera from CFA//TTd/Al-immunized mice. Furthermore, dissociation profiles of no//TTd/Al and no//TTd/glyc groups were similar, whereas corresponding CFA-pretreated groups showed statistically significant differences ($P < 0.0005$).

Anti- β_2 -GP I Abs

It has been shown that a variety of stimuli which increase the potency of T cell stimulation, including high Ag dose (20), can allow Th cells to escape regulatory T cell mediated suppression, leading to a break in peripheral tolerance. It has also been shown (26) that immunization of mice with multiple TTd doses ranging from 10–50 $\mu\text{g}/\text{mice}$ was not sufficient to induce experimental APS. For these reasons we decided to use 100 μg TTd/dose in our attempt to induce autoimmunity by hyper-immunization.

Concentrations of IgM and IgG specific for β_2 -GP I in individual mice sera were recorded during the course of immunization as well as during the post-immunization period; the mean concentrations at defined time points are presented in Figure 3. The decision to follow fluctuations in Abs specific for β_2 -GP I long after completion of immunization was based on the previously described mouse APS model, in which a long-term post-immunization increase in anti- β_2 -GP I IgG has been reported (27, 28). Additionally, the concentration of Abs specific for β_2 -GP I was examined in sera collected 1 week after the booster TTd dose application (check point C) to see whether memory cells specific for β_2 -GP I had been established.

Preliminary screening showed the presence of IgM and IgG specific for β_2 -GP I in normal mouse sera, but

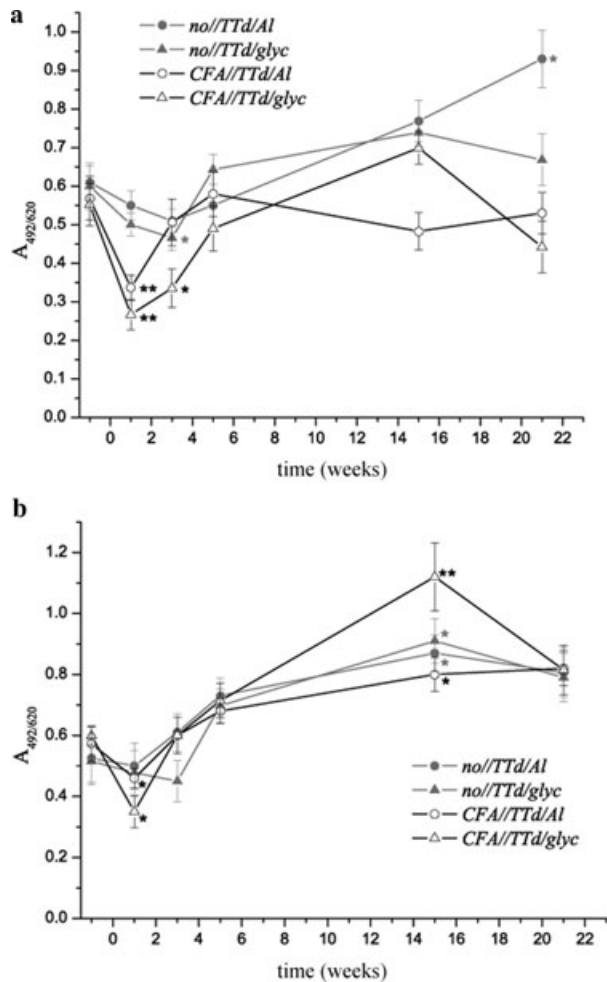


Fig. 3. Immunization-induced changes in the pool of IgM (panel a) and IgG (panel b) specific for β_2 -GP I. The reactivity of Abs belonging to the same isotype towards β_2 -GP I was examined simultaneously in individual sera. Results are presented as the mean $A_{492/620} \pm$ SE for each group ($n = 15$ mice/group) at defined time points. The statistical significance of immunization-induced fluctuations was determined by a paired Student's *t*-test using the $A_{492/620}$ for corresponding normal sera (-1 week) as a reference ($P < 0.05^*$, $P < 0.005^{**}$). Cut-off values for detection of IgM and IgG antibodies specific for β_2 -GP I were 0.03 and 0.07, respectively.

neither pretreatment (CFA) nor adjuvants (Al or glyc) per se induced an increase in them. In addition, analysis of *nc* group sera, as in the case of anti-TTd Abs, did not show any significant time-dependant fluctuations within IgM and IgG pools specific for β_2 -GP I.

Anti- β_2 -GP I IgM

Mean concentrations of anti- β_2 -GP I IgM in mouse sera collected at the main check points and during the course of immunization are presented in Figure 3a. The results show a transitional fall in IgM specific for β_2 -GP I during

the application of all immunization regimes. Compared with corresponding normal sera, observed decreases in concentrations of anti- β_2 -GP I IgM were statistically significant in the no//TTd/Al ($P < 0.05$ after the second TTd dose) and CFA-pretreated ($P < 0.005$ after the first TTd dose) groups. During the post-immunization period, an increase in IgM specific for β_2 -GP I was recorded in all groups except the CFA//TTd/Al, where a slight fall was observed. The booster TTd dose induced an increase in the anti- β_2 -GP I IgM in no//TTd/Al and CFA//TTd/Al groups, whereas in the TTd/glyc-immunized groups the concentrations of Abs specific for β_2 -GP I were diminished.

Anti- β_2 -GP I IgG

Measurements of anti- β_2 -GP I IgG concentrations in individual mouse sera (Fig. 3b) revealed a transitional fall during immunization of both pretreated groups. However, after completion of all immunization protocols, the concentrations of serum IgG specific for β_2 -GP I were higher than those in the corresponding sera taken before any treatment. During the post-immunization period, an additional increase in anti- β_2 -GP I IgG was observed in all groups and was more pronounced in TTd/glyc-immunized mice compared with the corresponding TTd/Al group. For all groups, maximal concentrations of IgG specific for β_2 -GP I, which were significantly higher than in the sera collected prior to any intervention, were detected in samples collected between the 7th and 11th week of the post-immunization period. Moreover, the concentrations registered within this interval were not significantly different. Respective to this observation, the 11th week of the post-immunization period was assigned as one of the main check points.

All further described analyses of the anti- β_2 -GP I IgG pool were performed at main check points only, with the use of each group's normal sera as well as A, B or C pools as samples.

Fine epitope specificity of anti- β_2 -GP I IgG

To assess the sera Abs' fine epitope specificity we simultaneously measured serum IgG reactivity with β_2 -GP I coated directly onto MaxiSorp plates (β_2 -GP I system) and with β_2 -GP I bound to immobilized PS (PS+ β_2 -GP I system).

On the basis of the results of our preliminary experiments, which showed the same reactivity of commercially available mouse anti-human β_2 -GP I IgG1 (clone 5F7) in both systems, we used this Ab as a control for coating equal amounts of β_2 -GP I ($A_{492/620}[\text{PS}] = 0.157 \pm 0.001$; $A_{492/620}[\text{PS} + \beta_2\text{-GP I}] = 2.003 \pm 0.044$; $A_{492/620}[\beta_2\text{-GP I}] = 1.993 \pm 0.044$).

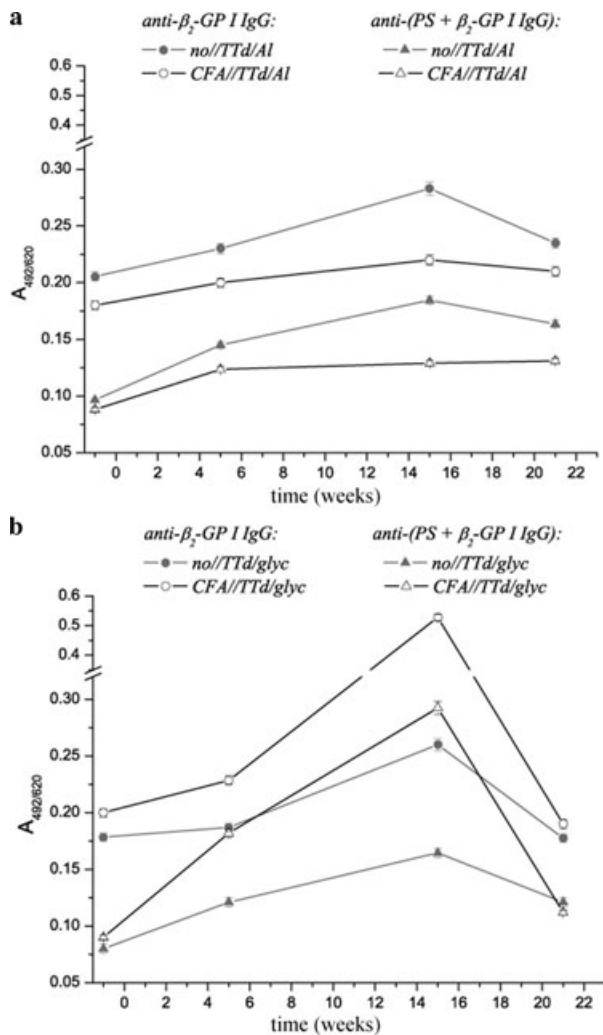


Fig. 4. Reactivity of IgG from sera of non- and CFA- pretreated mice immunized with TTd/Al (panel a) or TTd/glyc (panel b) toward β_2 -GP I (circles) and PS+ β_2 -GP I (triangles). For each group ($n = 15$ mice/group), normal, A, B and C sera pools were used as samples. Analysis of binding to β_2 -GP I was done simultaneously in β_2 -GP I and PS+ β_2 -GP I systems for all samples. All samples were assayed in triplicate and mean $A_{492/620} \pm$ SE are presented. Cut-off value for detection of anti- β_2 -GP I IgG in both systems was 0.07.

Reactivity of serum IgG toward β_2 -GP I in systems comprising β_2 -GP I and PS+ β_2 -GP I is presented in Figure 4. According to results for all groups at each time point, the reactivity of serum IgG was better for β_2 -GP I adsorbed directly onto the microplate. We approximated that the reduction of $A_{492/620}$ (PS+ β_2 -GP I) compared with the corresponding $A_{492/620}$ (β_2 -GP I), calculated as a percentage, was inversely correlated to the contribution of PS+ β_2 -GP I-specific IgG to the overall anti- β_2 -GP I IgG pool.

For the pools of normal sera, the reduction of $A_{492/620}$ values in the PS+ β_2 -GP I system, compared with the β_2 -

GP I system, was $55.08\% \pm 0.56\%$. At check point A, the reduction of $A_{492/620}$ in the PS+ β_2 -GP I system was less pronounced than for the pools of normal sera, indicating the prevalent synthesis of Abs specific for β_2 -GP I bound to immobilized PS. $A_{492/620}$ (PS+ β_2 -GP I) reductions were greater in the TTd/Al groups (36.96% for no//TTd/Al, 35.00% for CFA//TTd/Al) (Fig. 4a) compared with the TTd/glyc group that underwent the same pretreatment (35.29% for no//TTd/glyc, 20.35% for CFA//TTd/glyc) (Fig. 4b). During the 11-week post-immunization period the contribution of IgG specific for PS+ β_2 -GP I to the overall anti- β_2 -GP I IgG pool remained unaffected in non-pretreated groups (reductions at check-point B were 34.81% for no//TTd/Al and 36.73% for no//TTd/glyc). In contrast the CFA-pretreated groups showed a lowering tendency in anti-PS+ β_2 -GP I IgG (reductions at check-point B were 38.86% for CFA//TTd/Al and 44.60% for CFA//TTd/glyc). Finally, although application of the TTd booster dose did not induce an increase in total IgG specific for β_2 -GP I in either group, the contribution of Abs specific for PS+ β_2 -GP I was higher than that in the corresponding pool of normal sera. For sera pools at check point C, $A_{492/620}$ values in the PS+ β_2 -GP I system were from 30.43% (no//TTd/Al) to 47.05% (CFA//TTd/glyc) lower than corresponding values in the β_2 -GP I system.

Subclass analysis of anti- β_2 -GP I IgG

Preliminary screening showed that, irrespective of the applied immunization protocol, the contribution of individual subclasses to total anti- β_2 -GP I IgG was in the following order IgG1 > IgG2a > IgG3. The focus was on IgG1 and IgG2a anti- β_2 -GP I Abs with respect to 1) their contribution to the overall anti- β_2 -GP I IgG pool; 2) the fact that they could be regarded as markers of Th1/Th2 skewing (24) and 3) their already documented role in development of pathology related to anti- β_2 -GP I Abs (29). The results (Fig. 5) showed the following: First, an increase in both IgG1 and IgG2a Abs specific for β_2 -GP I was induced by application of all immunization protocols but with specific, context-dependent kinetics. Second, the increase in IgG1 anti- β_2 -GP I Abs was more pronounced during the course of immunization compared with the post-immunization period (Fig. 5a) when the increase of IgG2a anti- β_2 -GP I Abs was predominant (Fig. 5b). Furthermore, an increase in IgG2a Abs specific for β_2 -GP I was greater in the TTd/glyc-immunized groups compared with the corresponding TTd/Al group (Fig. 5b). Finally, the booster TTd dose did not induce an increase in either IgG1 or IgG2a Abs specific for β_2 -GP I (Fig. 5).

Affinity of anti- β_2 -GP I IgG

The dissociation profiles of β_2 -GP I/anti- β_2 -GP I IgG complexes in the presence of different KSCN

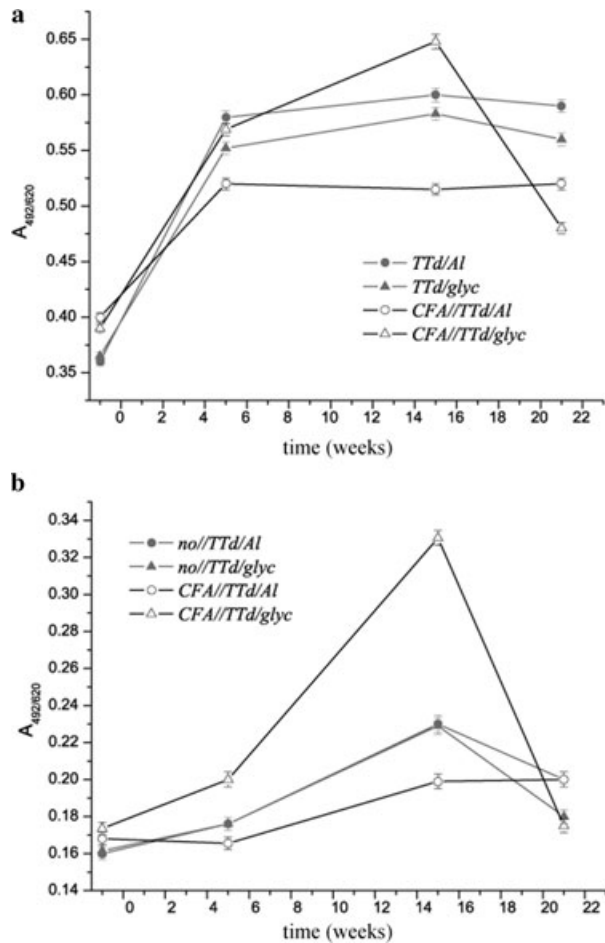


Fig. 5. Immunization-induced changes in the pool of IgG1 (panel a) and IgG2a (panel b) specific for β_2 -GP I. The reactivity of antibodies belonging to the same IgG subclass towards β_2 -GP I was examined simultaneously by using normal, A, B and C sera pools for each group ($n = 15$ mice/group) as samples. All samples were assayed in triplicate and mean $A_{492/620} \pm SE$ are presented. Cut-off values for detection of IgG1 and IgG2a specific for β_2 -GP I were 0.07 and 0.05, respectively.

concentrations were used for estimation and comparison of mean sera anti- β_2 -GP I IgG affinity toward β_2 -GP I. Comparison of corresponding dissociation profiles and $[SCN^-]_{50\%}$ values for normal and check point A pools, calculated for each pool upon linearization ($R > 0.96$), showed that completion of all immunization protocols induced synthesis of anti- β_2 -GP I IgG with higher affinity ($P < 0.005$) toward β_2 -GP I. Mean $[SCN^-]_{50\%}$ for normal sera pools was 3.81 ± 0.3 M. In contrast to the anti-TTd IgG, the mean affinities of anti- β_2 -GP I IgG, estimated according to $[SCN^-]_{50\%}$, were higher in sera from TTd/glyc-immunized mice ($[SCN^-]_{50\%}$ no//TTd/glyc = 4.98 M; $[SCN^-]_{50\%}$ CFA//TTd/glyc = 6.01 M) compared with the TTd/Al group under the same pretreatment protocol ($[SCN^-]_{50\%}$ no//TTd/Al = 4.85 M; $[SCN^-]_{50\%}$

CFA//TTd/Al = 4.93 M). Finally, a statistically significant difference in dissociation profiles between identically pretreated groups was registered only after CFA pretreatment ($P < 0.0005$).

Anti-laminin IgG

Simultaneous analysis of pools for each group comprising sera collected at 2-week intervals showed that in all groups, except CFA//TTd/Al, slight fluctuations within the anti-laminin IgG pool were induced (Fig. 6a). In the CFA//TTd/Al group, more intensive oscillations of anti-laminin IgG were detected. In the curve representing time-dependent oscillations of the anti-laminin IgG level, two peaks were noted in the CFA//TTd/Al group—in sera pools A and B. Analysis of reactivity toward laminin performed for individual normal sera, as well as sera collected at check points A and B, confirmed that the concentration of anti-laminin IgG found 1 week after completion of immunization was significantly greater than that in normal sera ($P < 0.05$).

Natural antibodies

The influence of the described TTd immunizations on the natural Abs pool was estimated according to time-dependent fluctuations in the expression of the natural idiotope Y7 on the sera IgM (30, 31) (Fig. 6b).

Given that natural Ab concentrations have a wide physiological range (mean PI \pm SE of normal sera pools was $52.81 \pm 8.99\%$) and considerable time-dependent oscillations (PI range for *nc* was 19.8%), all fluctuations within a range less than 20% were not considered significant. In accordance with Y7 expression, the most intensive fluctuations were recorded in the CFA//TTd/Al group (PI range was 48.91%) (Fig. 6b). In that group two pronounced falls in serum Y7+ Ig concentration were registered: the first after completion of immunization and the second after the booster TTd dose. For all other groups, the PI range was less than 20%.

Evaluation of pathology related to anti- β_2 -GP I Ab

The analysis of pregnancy outcomes (fecundity, number of fetuses and fetal resorption rate) following TTd-immunization was used for evaluation of the pathogenic potential of induced immune responses. This was based on the following: 1) the role of anti- β_2 -GP I Abs in genesis of pregnancy-related pathology has been well documented (18) and 2) secretion of anti- β_2 -GP I Abs was induced in all immunized mice in our experiment.

Results showed that pregnancy outcomes were significantly context-dependent, implying different pathological

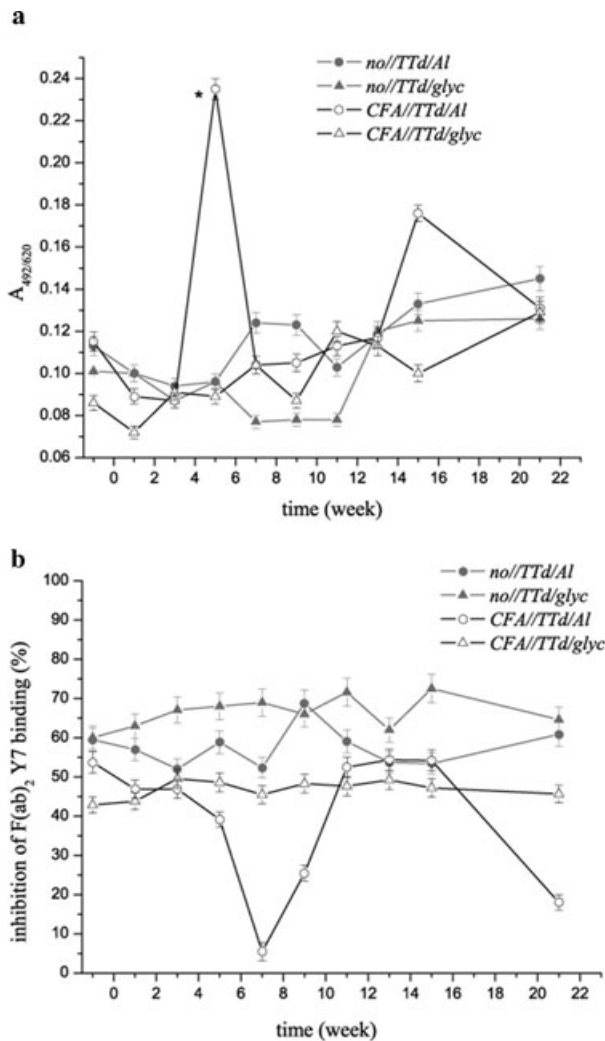


Fig. 6. Immunization-induced changes in reactivity of sera IgG with mouse laminin (panel a), and fluctuations within the natural Ab pool followed via expression of Y7 idiotope (panel b). The expression of Y7 idiotope was measured through inhibition of F(ab)₂ Y7-B binding to IgM DJ (Y7⁺ IgM) adsorbed onto plastic in the presence of defined sera pools ($n = 15$ mice/group). All samples were assayed in triplicate and mean $A_{492/620} \pm SE$ (panel a) or mean PI $\pm SE$ (panel b) are presented. Cut-off value for detection of anti-laminin IgG was 0.08.

potentials for the induced immune responses. The results from two independently performed experiments are presented in Table 1. A slight decrease in fecundity (number of the pregnant females) following TTd-immunization, which was most pronounced in the no//TTd/Al group, was observed.

Analyses of placentas and fetuses were performed on the 14th day of pregnancy. A reduction in the total number of fetuses (defined as the sum of NoF and NoFR), as well as an increased rate of fetal resorption (defined as a more than 50% reduction in fetal volume), was observed. The most

Table 1 Evaluation of pregnancy outcomes in TTd-immunized mice performed on the 14th day of gestation

Treatment type	Pregnant mice (%) [†]	Total number of fetuses [‡]	Fetal resorption [‡] (%)
nc	75.00 \pm 5.00	9.22 \pm 0.54	3.78 \pm 1.02
no//TTd/Al	61.77 \pm 2.3	9.01 \pm 0.39	8.58 \pm 2.91
no//TTd/glyc	66.33 \pm 3.3	7.02 \pm 0.22*	5.72 \pm 2.08
CFA//TTd/Al	66.45 \pm 4.55	6.43 \pm 0.52*(a)	16.67 \pm 3.13*(a)
CFA//TTd/glyc	71.71 \pm 3.29	5.33 \pm 0.31**	7.53 \pm 2.56

[†]Mean value calculated from two independent experiments (8–10 mice per group per experiment).

[‡]Mean fetus number/fetal resorptions \pm SE per pregnancy, calculated for all mice immunized according to same protocol in two independent experiments.

* $P < 0.05$, ** $P < 0.005$ (t -test for independent groups; nc group was used as the reference).

^a $P < 0.05$ (comparison of CFA-pretreated groups by t -test for independent groups).

striking reduction in number of fetuses was detected in CFA//TTd/glyc-immunized mice. Statistically significant decreases in the total number of fetuses were also noticed in the no//TTd/glyc and CFA//TTd/Al groups. When comparisons between the experimental groups that underwent the same pretreatment were performed, a greater fetal resorption rate was found in TTd/Al-immunized mice. However, compared with nc mice, only the CFA//TTd/Al group showed statistically significant increases in fetal resorption. One must note that, in addition to fetal resorption, placental hemorrhages, accompanied by a lesser degree of reduction in fetal volume, were seen in immunized pregnant mice but not in nc pregnant mice.

DISCUSSION

Our current research has confirmed that TTd could be an inducible target for production of Abs specific for β_2 -GP I (26) and, more importantly, has described modulation of the self-reactive immune response in the context of TTd application, which eventually results in the response having different pathological potentials.

In this paper, we have reported on three sets of changes in BALB/c mice sera induced by immunization with high concentrations of TTd: firstly, changes induced in profiles of sera IgM and IgG specific for TTd; secondly, changes in profiles of autoAbs specific for β_2 -GP I and mouse laminin; thirdly, changes in the expression of Y7 natural idiotope.

It is clear that the induced general immune response is a result of interplay between the pretreatments and the impact of the adjuvants. CFA is a mixture of TLR agonists (lipid A, N-acetylmuramyl-L-alanyl-D-isoglutamine, trehalose-6,6-dimycolate and CpG) (25)

and possesses a high activation potential. Through its action on TLR-2, TLR-4 and TLR-9, CFA activates B and T cells. Owing to its chemical chaperone potential, glycerol primarily stimulates T cells (24) while Al is cited in the literature as a B cell stimulator (23).

Although our focus was on the properties of modulation of self-directed immune responses, we also analyzed the TTd-specific immune response in order to reveal the relationship between self-directed and Ag-specific immune response. In all hyper-immunized mice, irrespective of the immunization protocol applied, TTd-specific Abs were induced. The fact that hyper-immunization does not suppress Ag-specific immune response (32) but, on the contrary, induces its strong stimulation can be explained by the Th2-predominant genetic background of BALB/c mice (33) and thus predominant generation of a Th2 response. Th2 clones, compared to Th1 clones, are more resistant to activation-induced cell death triggered by high doses of antigens (34). Relative to the time-dependent changes of anti-TTd IgG concentrations, TTd immunization resulted in either a classic adaptive response (TTd/glyc immunizations) or in the persistence of high concentrations of serum Abs (TTd/Al immunizations). Moreover, a comparison of the groups pretreated in the same way indicates that use of Al as adjuvant has a positive impact on anti-TTd IgG affinity.

Concurrent with the TTd-directed immune response, a self-reactive immune response was also induced in all groups. The anti- β_2 -GP I Abs detected after completion of immunization could be the result of perturbation within the naturally occurring Abs pool or a part of the TTd-specific Abs generated from molecular mimicry. Because of structural homology between TTd and β_2 -GP I, the observed post-immunization increase in IgG with specific affinity for β_2 -GP I indicates that a T cell-dependent component of the self-reactive response is most probably activated. Moreover, curves of the same shape, as observed in the TTd/Al groups, represent fluctuations in Abs specific for TTd and β_2 -GP I and stress the importance of the overlap between these Abs. In contrast, the glycerol-induced segregation of the humoral immune response specific for TTd and β_2 -GP I may be a consequence of a pronounced Ag-specific T cell-dependent branch in both TTd/glyc groups.

When we consider anti- β_2 -GP I Abs, the following facts must be kept in mind (18): 1) Anti- β_2 -GP I Abs occur in normal sera as part of the natural Ab pool exerting a homeostatic role. 2) Anti- β_2 -GP I Abs comprise a mixture of Abs specific for constitutively exposed epitopes on the surface of β_2 -GP I, as well as Abs specific for cryptic epitopes (35) uncovered following β_2 -GP I adsorption to a negatively charged surface (like PS). 3) Effector functions and pathological potential of anti- β_2 -GP I Abs

depend on their concentration, affinity and fine epitope specificity.

Correlation of pregnancy outcomes with the results of performed serological tests implies that concentration of total anti- β_2 -GP I Abs per se is an important (as concentration of total anti- β_2 -GP I Abs was higher following immunization than in normal sera) but not the primary factor in pathology induction (the lowest level was recorded in the CFA/TTd/Al group). Our results show that the severity of pathology can be correlated to parameters which could be connected to direct Abs binding: 1) the abundance of IgG that recognized β_2 -GP I following interaction with PS and 2) IgG affinity. Although a clear correlation between the concentration of complement fixing Abs (IgG2a and IgM) and severity of pathology was not observed, the greatest abundance of IgG2a within the overall anti- β_2 -GP I IgG (revealed according to $A_{492/620} [\text{IgG1}]/A_{492/620} [\text{IgG2a}]$) in the sera of CFA/TTd/glyc mice implies their importance in genesis of pathology. In addition, given that IgG2a is a hallmark of Th1 response (24), it would seem that skewing of the immune response in a Th1 direction would be more deleterious.

By analyzing the mutual relationship of serum IgG reactivity in β_2 -GP I and PS+ β_2 -GP I systems we approximated the concentration of Abs specific for cryptic epitopes on β_2 -GP I. These Abs could account for smaller differences between $A_{492/620}$ values obtained in the β_2 -GP I and PS+ β_2 -GP I systems for sera pools A, B and C than in corresponding normal sera pools. We assumed that observed pregnancy pathology was a result of induced anti-PS+ β_2 -GP I Abs targeting trophoblast (36) and fetus (37). Apoptosis as a normal process during placental formation (38) and fetal development (37) lead to the creation of one PS-rich surface that allows formation of numerous PS+ β_2 -GP I complexes. Furthermore, it has been documented that numerous trophoblast properties and functions are negatively affected (defective invasiveness and hormone secretion, impaired embryonic implantation, infarctions) by direct anti- β_2 -GP I Abs binding and/or concomitant complement activation, resulting in various complications of pregnancy (reduced fecundity and number of fetuses, increased fetal resorption rate) (18, 39–41).

The necessity to use β_2 -GP I in quite a high concentration (10 $\mu\text{g/ml}$) for detection of anti- β_2 -GP I Abs, thus allowing its multivalent binding, implies that these Abs recognize specific Ags with low affinity. Comparison of $[\text{SCN}^-]_{50\%}$ for normal sera pools and for A sera pools shows that all immunization protocols induced an increase in mean affinity of anti- β_2 -GP I/ β_2 -GP I interactions, this being higher in CFA-pretreated groups. However, as we measured affinity in a system using a high-salt concentration, we must remember anti-cryptic epitope Ab/cryptic

epitope interactions. Namely, it has been demonstrated that not only interaction of β_2 -GP I with a negatively charged surface, but also high ionic strength, can expose cryptic epitope on β_2 -GP I in solution (42). So, it may be more correct to say that higher $[\text{SCN}^-]_{50\%}$ values for A sera pools are a consequence of affinity maturation within the anti- β_2 -GP I IgG pool and/or more abundant IgG specific for cryptic epitopes on β_2 -GP I than in normal sera.

A complex Ag that includes an adjuvant could be expected to promote all forms of immune responses (innate, T dependent, T independent), with each response having a specific contribution. The increase in concentration of IgM detected after the TTd boost implies activation of innate immunity-based T cell-independent response in all groups except CFA//TTd/glyc. However, the intense fluctuation in the expression of natural Y7 idiotope shows that its involvement in shaping of overall immune response is most pronounced in the CFA//TTd/Al group. In regard to the reduction in number of fetuses observed in the CFA-pretreated groups, involvement of innate immunity has a positive impact, making an overall immune response less deleterious. However, in regard to the high resorption rate observed in the CFA//TTd/Al group, it could be hypothesized that epitope spreading and affinity maturation within the natural Abs pool results in a harmful immune response. Furthermore, it is interesting to compare time-dependent fluctuations within the anti-laminin IgG pool with Y7 expression. A decrease in percentage of Y7+ natural Abs was detected immediately after an increase in the concentration of IgG specific for mouse laminin, and coincided with an increase in anti- β_2 -GP I IgG. Noting that anti-laminin Abs possess a high pathological potential in murine systems (17), one could regard the decrease in natural Y7 idiotope expression as an attempt by the system to roll back to a steady state, using a natural Abs pool as the basis. However, the suppression of one autoimmune response (anti-laminin Abs) was accompanied by activation of another one (anti- β_2 -GP I), a feature that has been referred to as the “kaleidoscope of autoimmunity” (43).

We have convincingly demonstrated that the border between autoimmunity and overt autoimmune disease is very narrow, and is dependent on the context of the triggering Ag application. Molecular mimicry, which results in generation of anti- β_2 -GP I Abs on TTd immunization, is necessary, but not sufficient in itself, for the development of severe pathology. The existence of additional triggering events, as determined by the context of the Ag application, also seems to be necessary. In the context of APS, activation of T cells seems to be critical, since induction of statistically significant pathology was observed following TTd application in contexts which provided their activation (glycerol and/or CFA). Further-

more, the more pronounced pregnancy-related pathology in the CFA-pretreated groups implies a role of activation via TLRs.

We suggest that examination of immune responses induced by micAg applied in different contexts could be useful for defining additional signals and mechanisms which lead to autoimmunity-autoimmune disease conversion. Analysis of autoAbs pool characteristics (affinity, isotypic profile, fine epitope specificity and concentration) could be useful for prediction of the pathological potential of induced immune responses. Finally, in the context of an APS study, this model could be regarded a new model system. Although the importance of micAg in APS etiology has been clearly demonstrated, currently described models are based on APS induction by β_2 -GP I, peptides that share structural homology with β_2 -GP I or specific Ab immunization (reviewed in 44).

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