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Lack of patent liver autoimmunity after breakage of tolerance in a mouse model

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Abstract

We report in this work that a cellular and humoral autoreactive response can be induced against liver-specific self-determinants by repeated immunization with a chimeric tissue-specific self-antigen carrying a heterologous T_h epitope. Epitope spreading rendering the autoimmune reaction independent of the presence of the cognate heterologous help is also demonstrated. Although neutrophil infiltrates can be demonstrated in the livers of treated mice, no clinical sign of organ damage is observed. These findings suggest that breakage of tolerance by this means leads the process only up to the next checkpoint in the progression of autoimmune disease and that further events are required to precipitate functional organ impairment.

Introduction

Autoimmunity is an evolving process in which autoimmune responses shift in time not only against other determinants of the initiating antigen, but also against other self-antigens (1–4). Determinant spreading, leading to the expansion of cohorts of autoreactive cells, is thus the hallmark of such a pathogenetic mechanism (3). How autoreactivity against a self-epitope is triggered and how it spreads to other determinants of the same or of other self-antigens, and what parameters can precipitate such drifting and lead to disease, is less clearly understood (5). For example, in autoantibody animal models, whether transgenic or otherwise obtained, escape of autoreactive B cells from clonal deletion or anergy alone is not sufficient to allow the development of a B cell-dominant autoimmune disease, while additional factors such as infections, cytokines, help by T cells and others are required (6–8). Although the correlation may not be universally valid (9), its support to the hypothesis that T cell help and T and B cell cooperation are important for the onset of certain autoimmune conditions derives from many studies where model animals are immunized with modified self-antigens or with cross-reacting antigens mimicking self.

Immunization with a self-protein normally fails to stimulate antigen-specific T or B cells because of the establishment of tolerance mechanisms at the thymic or peripheral level which make any potential autoreactive determinant invisible to the immune system. Such a state of unresponsiveness to self-antigen, however, is not absolute as breakage of tolerance can be attained by different approaches and at different levels. For example, mice immunized with synthetic peptides derived after the primary sequence of a self-protein can activate unscheduled peptide-specific T_h cells, suggesting that ignorance rather then tolerance may play a role in some cases (10). Similarly, it is possible to activate an autoantibody response by using a modified self-protein as an antigen (10–14). Breaking of B cell tolerance towards a conserved self-protein such as ubiquitin has been achieved by inserting a foreign T cell epitope (11,12). Tolerance may also be broken by using cross-reactive foreign antigens, such as cytochrome *c*, having limited sequence diversity to the murine protein (10). The latter reports illustrate how B cell responses to self are limited by lack of appropriate T cell help. The question arises as to whether determinant spreading occurs as a secondary response to activation of T cells by the heterologous epitope inserted into the chimeric self-antigen. Dalum *et al.* (12) reported that if determinant spreading occurs in their system, it is only associated with and maintained by the exogenously administered modified antigen carrying the foreign determinant. In fact, in their case, a decline in the autoantibody titer occurred after suspension of immunization with modified ubiquitin, whilst the presence of endogenously produced ubiquitin would have suggested the possibility of a continuous stimulation of autoantibody-producing B cells and thus progression towards a true autoimmune status. However, induction of tissue damage or other cytopathic effects were

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not reported in animals in which autoantibody production was triggered by inoculation of a modified self-antigen carrying heterologous help. On the other hand, as the self-antigens used in these cases (ubiquitin and cytochrome *c*) were ubiquitously expressed in the body of the animals, tissue damage was not examined in specific target tissues. Therefore the question could not be resolved as to whether breakage of tolerance by means of immunization with modified or cross-reacting ubiquitous self-antigen can precipitate the onset of an autoimmune syndrome in the absence of other inducing factors. To further investigate this aspect, we chose in this paper to use the murine α glutathione-S-transferase (mGST) as a self-antigen. In fact, the expression of mGST is restricted to a few sites, and particularly to the liver and lymphocytes, therefore allowing study of the effects of breaking of tolerance in a specific target organ. GST protein isoforms and genes from different species have the additional advantage of being very well known from a structural point of view, can be easily manipulate by insertion of a foreign helper epitope and can be rapidly purified to homogeneity. Furthermore, a well-characterized mixed helper/B cell epitope, pep23, was chosen to provide heterologous help on the basis of its ability to induce a response in BALB/c animals; the latter strain was in turn used because there is no evidence in the literature that it may spontaneously develop a liver-specific autoimmune disease. Our results indicate that, under our conditions, immunized animals develop anti-mGST autoantibodies, epitope spreading to self-determinants and inflammatory cell infiltration, but no signs of the onset of clinical autoimmune symptoms.

Methods

Animal experiments

All animals received care according to the regulations set by the Italian Ministry of Health, and fulfilling EU Directives 86/609/EEc and 90/67/EEC.

Immunogen purification

The pRTC99a expression prokaryotic vector (Amersham Pharmacia Biotech, Milan Italy) was used to clone in the *Kpn*I–*Bgl*II sites of the mGST cDNA. This cDNA was obtained using the mRNA prepared from mouse adult liver of the BALB/c strain with two oligonucleotides specific for the α class mGST: 5′-ggtaccatggcggggaagccagtccttca-3′and 3′-cagcaaagaaattttcagttaaagatct-5′.

To clone the pep23 synthetic peptide (KDSWTVNDIQKLVGK), corresponding to residues 248–262 of HIV-1 reverse transcriptase at the N- and C-terminal position of mGST, double-strand synthetic oligonucleotides were inserted respectively in either the *Eco*RI–*Kpn*I or *Bgl*II–*Xba*I site of pTRC99A. The constructs were used to transform *Escherichia coli* Sure strain and the recombinant proteins were purified with the same procedure suggested by Amersham Pharmacia Biotech for *Schistosoma japonicum* GST purification, using a GST-affinity column. After binding of the induced protein to the column, the protein was eluted using reduced glutathione. The purified proteins were analyzed by SDS–PAGE on 15% gels and stained with Coomassie blue. Recombinant protein concentration was measured with the Bio-Rad (Milan, Italy) protein assay.

Mice immunization

Female BALB/c (H-2^d) mice (6–8 weeks old) were obtained from Charles River (Milan, Italy) and housed at the IGB animal facility; sentinel mice were screened for seropositivity to Sendai virus, rodent coronavirus and *Micoplasma pulmonis* by the Murine Immunocomb test (Charles River), and were found negative.

Three groups of four animals were immunized with mGST carrying a peptide insert at the N-terminal (23mGST) or C-terminal (mGST23) position by an initial i.p. injection of 100 μg antigen solubilized in PBS and emulsified with an equal volume of complete Freud's adjuvant (CFA). Booster injections with the same amount of antigen emulsified 1:1 in incomplete Freud's adjuvant (IFA) were given i.p. on days 14 and 28. Blood samples were collected every 2 weeks after each injection. Serum were separated by centrifugation and stored at –20°C

ELISA assays

For detection of specific antibodies, polystyrene microtiter plates were coated overnight at 4°C with 2 μg/well in a volume of 50 μl with antigens dissolved in coating buffer (7.3 mM Na_2CO_3, 17.4 mM $NaHCO_3$ and 0.1 mg/ml NaN_3). Residual binding sites were blocked with 200 ml/well of 0.5% BSA in blocking buffer (20 mM Tris, pH 7.3 and 130 mM NaCl), and 100 μl/well of serum dilutions (diluted in 0.25% BSA, 20 mM Tris, pH 7.3, 0.5M NaCl and 0.05% Tween 20) was added to each well and incubated for 1 h at room temperature. After washing twice with EWB buffer (20 mM Tris, pH 7.3, 130 mM NaCl and 0.05% Tween 20) and twice with TBS buffer (20 mM Tris, pH 7.3 and 500 mM NaCl), 100 μl of horseradish peroxidase-labeled goat anti-mouse IgG (Sigma, Milan, Italy) diluted 1:2000 in washing buffer containing 1% BSA was added to each well and incubated for 1 h at room temperature. The binding was subsequently visualized with 1 μg/ml *o*-phenylenediamine substrate (Sigma) solution in 3.5 mM citrate, 7.5 mM Na_2HPO_4 (pH 5) containing 0.03% H_2O_2. The reaction was stopped by addition of 150 μl 2 N H_2SO_4/well and the absorbance at 492 nm was measured.

Proliferation test

BALB/c mice immunized as previously described were sacrificed 8 days after the last boost, and spleen and popliteal lymph nodes were removed aseptically.

Syngenic spleen cells were used as antigen-presenting cells (APC) and incubated overnight with different antigen concentrations in a same volume. Antigen-pulsed (or non-pulsed) APC (2.5×10^5) were then irradiated, washed, suspended in RPMI 1640/10% FCS, 5×10^{-5} M 2-mercaptoethanol, penicillin/streptomycin, 100 μM non-essential amino acids and 1 mM sodium pyruvate, and plated in 96-well microtiter plates with 2.5×10^5/well T cells from each mouse and cultured for 4 days. Proliferation responses were assayed by [^{3}H]thymidine incorporation (0.5 mCi/well) for 16 h and harvested with a Micromate cell harvester. The dry filters were counted in a Packard (Milan, Italy) β counter. Results were expressed as specific proliferative indices. Experiments were

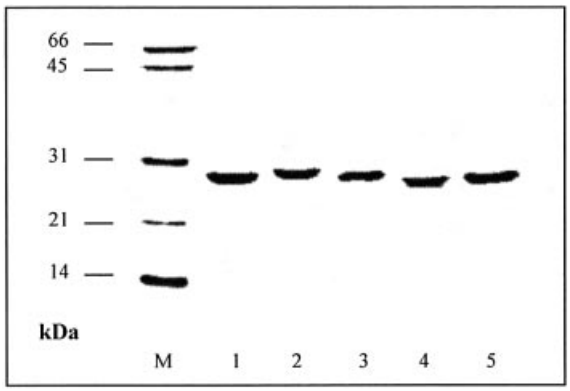

Fig. 1. SDS–PAGE analysis of recombinant proteins used in immunization experiments and ELISA assays. Gel lines show purified recombinant proteins: mGST (1), 23mGST (2), mGST23 (3), GST (4) and GST23 (5).

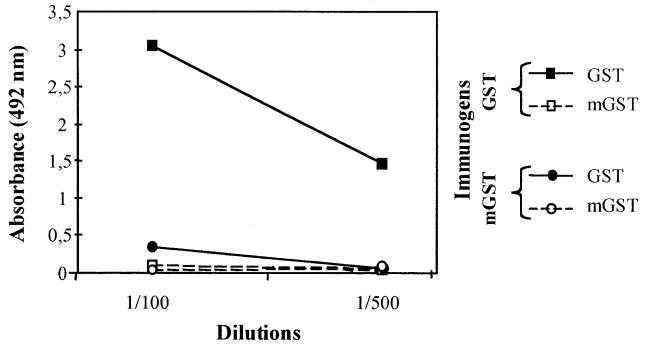

Fig. 2. Elisa titers for antisera from BALB/c mice immunized with GST from *S. japonicum* (squares) and with mGST (circles). Eight mice were immunized i.p. with 100 μg of antigen emulsified 1:1 in CFA, and three booster injections with the same amount of antigen in IFA were given i.p. on days 14, 28 and 42. Serum samples were collected 2 weeks after the last boost, diluted 1:100 and 1:500, and dispensed into wells coated with GST (filled symbols) or mGST (open symbols). Data represent the values for one mouse of each group.

independently performed at least 3 times for each sample and each time triplicate readings were taken.

Cytological analysis

Livers were removed and fixed in 10% neutral buffered formalin. After being mechanically rinsed, dehydrated and embedded in paraffin, the tissue was sectioned (5 mm) and stained with hematoxylin & eosin. Samples were coded and blindly prepared by a histologist and examined by a pathologist. Four 23mGST- and four mGST23-immunized animals producing various levels of anti-mGST antibodies were examined together with non-immunized animals or animals immunized with GST23 or 23GST and not responding to mGST. Several slides (three to five), each carrying a tissue section, were exhaustively examined under the microscope.

Statistical analysis

The statistical analysis was performed by Mann–Whitney non-parametric test with InStat software. The *P* value obtained for the reactivity against mGST is 0.03 and it is considered significant. The *P* value obtained for the reactivity against GST23 is 0.3 and is considered non-significant.

Results

Preparation of immunogens

An α class GST cDNA (15), derived by RT-PCR of mRNA from livers of adult BALB/c mice, using specific oligonucleotides derived from data bank sequences (GenBank accession no M73483), was cloned in the prokaryotic expression vector pTRC99A. After cloning, mGST protein was expressed in the *E. coli* cell cultures, following IPTG induction. The mGST protein expressed in the mouse liver shows 56% identity with respect to GST from *S. japonicum* and can be purified by using the same glutathione-affinity system commonly used to purify the protein from *S. japonicum* (16). The bacterial extracts containing mGST protein were thus loaded on a glutathione–Sepharose 4B column and, after affinity binding, the mouse protein was recovered by elution with a buffer containing reduced glutathione. Figure 1 shows a SDS–PAGE analysis of the recombinant proteins after purification. GST from *S. japonicum* (Fig. 1, lane 4) and mGST from mouse (Fig. 1, lane 1) were then used for mice immunizations and ELISA assay.

In order to study the role of a single foreign T_h epitope in the immunogenicity of recombinant proteins, two different mGST fusion proteins were produced, both containing the same foreign peptide, i.e. pep23 (KDSWTVNDIQKLVGK), corresponding to the amino acid sequence 248–262 of the HIV-1 reverse transcriptase. This peptide carries a T_h epitope recognized in a MHC A^d-restricted fashion in BALB/c mice (17). A double-stranded oligonucleotide codifying the pep23 was cloned in-frame upstream or downstream to the mGST cDNA in pTRC99a as described in Methods. These constructs were expressed in *E. coli* cells, upon IPTG induction, to obtain mGST recombinant proteins carrying the pep23 foreign peptide at the N- or C-terminal position of GST. The two proteins, identified as 23mGST and mGST23, were purified with the same affinity column used to purify mGST and analyzed by SDS–PAGE (Fig. 1, lanes 2 and 3).

Immunogenicity of mGST, 23mGST and mGST23

Two groups of four BALB/c mice were separately immunized i.p. with mGST and GST proteins respectively, and booster injections were given on days 14, 28 and 42. The reactivity of antisera toward mGST and GST was analyzed in ELISA using the same proteins immobilized on the plates. Mice receiving GST rapidly developed a significant anti-GST response, confirming that GST is a highly immunogenic molecule; these immunized animals do not show cross-reactivity with mGST. On the other hand, two groups of four mice immunized with mGST were unable to produce autoantibodies even after three consecutive boosts, demonstrating that recombinant mGST is non-immunogenic, as expected for a self-protein. Representative ELISA results obtained for one mouse among the group of eight receiving each protein are shown in Fig. 2.

On the other hand, mice receiving the self-protein fused to the foreign immunodominant epitope developed both an anti-mGST and an anti-peptide response, but no anti-GST

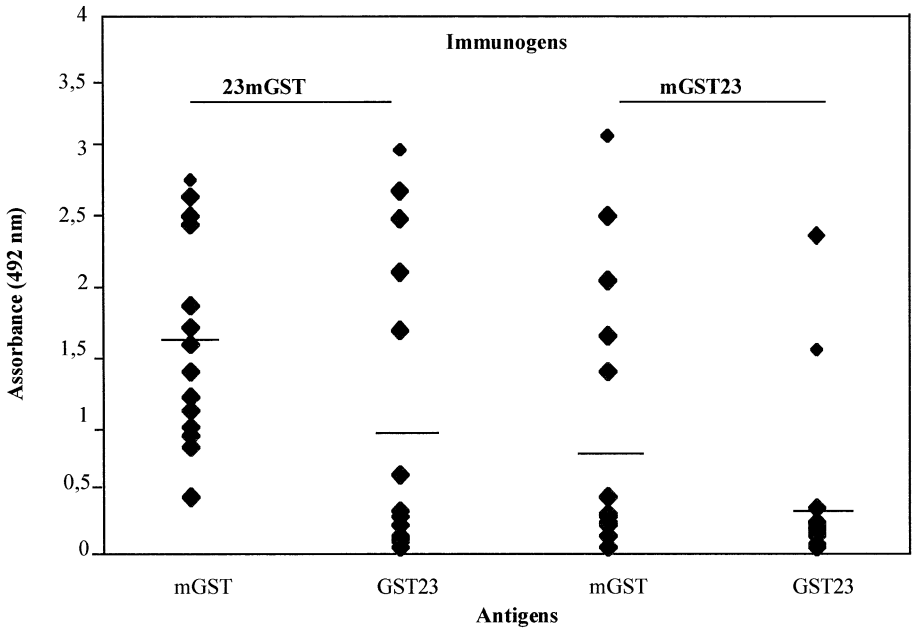

Fig. 3. ELISA titers for antisera from BALB/c mice immunized with 23mGST or mGST23. Twelve mice for each group were immunized i.p. with 100 μg of antigen emulsified 1:1 in CFA, and three booster injections with the same antigen in IFA were given i.p. on days 14, 28 and 42. Serum samples were collected 2 weeks after the last boost, diluted 1:500 and dispensed into wells coated with mGST or GST23. The horizontal bar indicate the data mean.

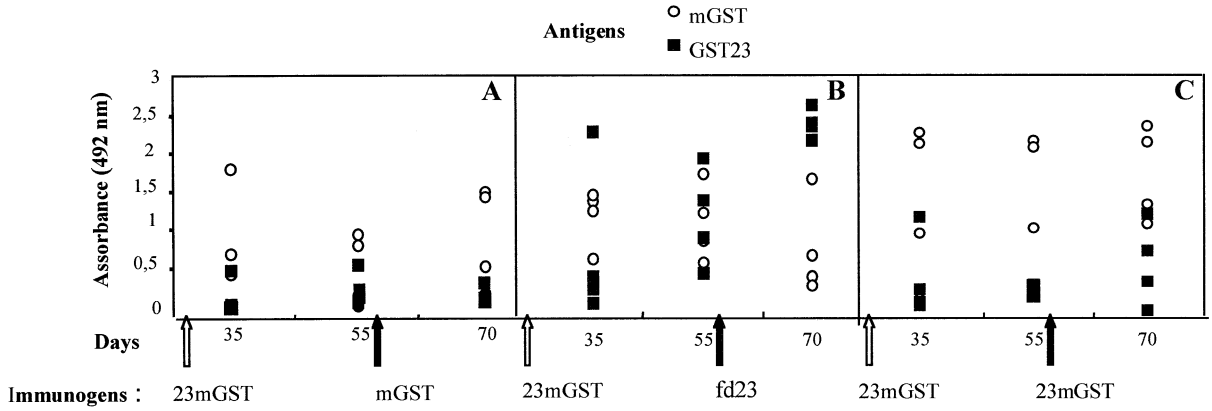

Fig. 4. ELISA titers for antisera from BALB/c mice after sequential immunization with 23mGST followed by boosts with the self-protein mGST (A), the same recombinant protein (C) and with recombinant bacteriophage fd23 (B). Twelve mice were immunized i.p. with 100 μg of 23mGST emulsified 1:1 in CFA and the booster injections with different antigens, shown in the panels, solubilized in IFA were given i.p. after 56 days. Serum samples were collected every 3 weeks to monitor the primary and secondary response, diluted 1:400, and dispensed into wells coated with mGST (open circles) or GST23 (filled squares). The arrows show the time of the injections and the immunogen administered.

reactivity. In fact, groups of mice were separately immunized i.p. with 23mGST and mGST23 proteins by an initial injection, and three booster injections delivered i.p. at 2, 4 and 6 weeks. Sera were collected 2 weeks after the last boost and all assays were performed using 1/500 dilutions of the sera. The reactivity of antisera from all mice against unmodified mGST and against the peptide pep23 was analyzed in ELISA using immobilized mGST (for self-epitopes) and GST23 (to test the pep23 epitope) proteins (18). Figure 3 shows that mice receiving 23mGST or mGST23 developed a titered anti-mGST and anti-pep23 response indicating that insertion of the pep23 epitope at the N- or C-terminal position makes the self mGST molecule immunogenic. Immunization of a group of four mice with a mixture of mGST and synthetic pep23 in equimolar amounts according to the same schedule used to immunize against mGST23 or 23mGST in no case caused induction of anti-mGST self-antibodies (<0.05 OD_{492}). The level of antibody responses observed was variable in different mice and was almost absent in some of them. In addition, a statistically significant lower antibody response was observed in mice immunized with mGST23, the protein carrying the foreign epitope at the C-terminal position of the mGST, than in mice with 23mGST, the protein carrying the foreign epitope at the N-terminal position of the mGST.

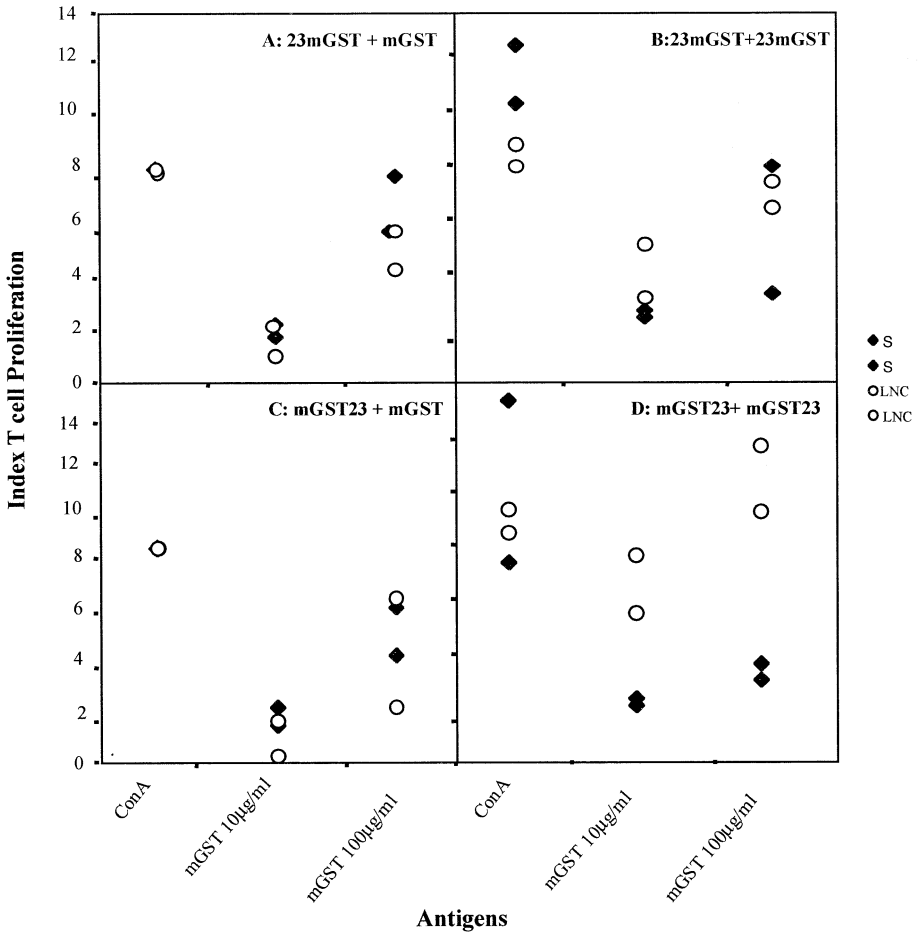

Fig. 5. T cell-dependent proliferative response of lymph node cells and splenocytes from mice immunized with recombinant proteins. BALB/c mice were immunized i.p with 100 µg of antigen emulsified 1:1 in CFA, and three booster injections with the same antigen in IFA were given i.p on days 14 and 28, and into both hind footpads on day 42. Mice were primed with 23mGST (A and B) or mGST23 (C and D) and re-stimulated with mGST (A and C) or with the same protein used during the first boost (C and D). T cells were *in vitro* re-stimulated with 10 or 100 µg/ml mGST. The results obtained for splenocytes are represented by diamonds and for lymph node cells by open circles. Concanavalin A (5 mg/ml) was used as positive control.

To assess whether the position of peptide in the protein context is important to determine the immunogenicity of the whole protein we performed a statistical analysis of the results by applying the non-parametric Mann–Whitney test to the two groups of data obtained for mGST and GST23 in ELISA assays. When the reactivity of the two groups of mice immunized with 23mGST and mGST23 against mGST was statistically compared, $P = 0.03$ was obtained. In contrast, when we analyzed the reactivity against GST23, $P = 0.3$ was found. The data demonstrate that the position of the peptide is not important in determining the immune response against the pep23 epitope, but is important in breaking tolerance against self-protein. When the foreign epitope is located at the N-terminal position, the determinant spreading probably occurs more efficiently and the antibody titer against mGST is higher.

Primary and secondary response of antisera

To further study the mechanism of tolerance breakage, we monitored the primary antibody response during the weeks following the first immunization and the secondary antibody response during the weeks following the boost. Twelve mice were immunized i.p. with 100 µg of 23mGST protein and 12 mice with mGST23. After the first inoculation, each mouse was bled at 14, 35 and 52 days from the orbital sinus to monitor the antisera reactivity before receiving a second injection. The second boost was administered differently to 12 mice of each group. In fact, four mice received the same protein used during the first injection (23mGST or mGST23), four mice were boosted with the unmodified mGST protein and the last four mice were re-stimulated with a different immunogen, the fd23 recombinant bacteriophage (19), carrying the same pep23 epitope present in the fusion protein. After the boost, each mouse was bled at 14, 35 and 52 days from the orbital sinus, and the ability of the raised antisera to react against native mGST and GST23 was tested in ELISA assays.

Figure 4 shows the reactivity of all antisera during the primary and secondary response obtained for the groups immunized with 23mGST. The results obtained upon immunization with mGST23 are not shown; however, the finding that

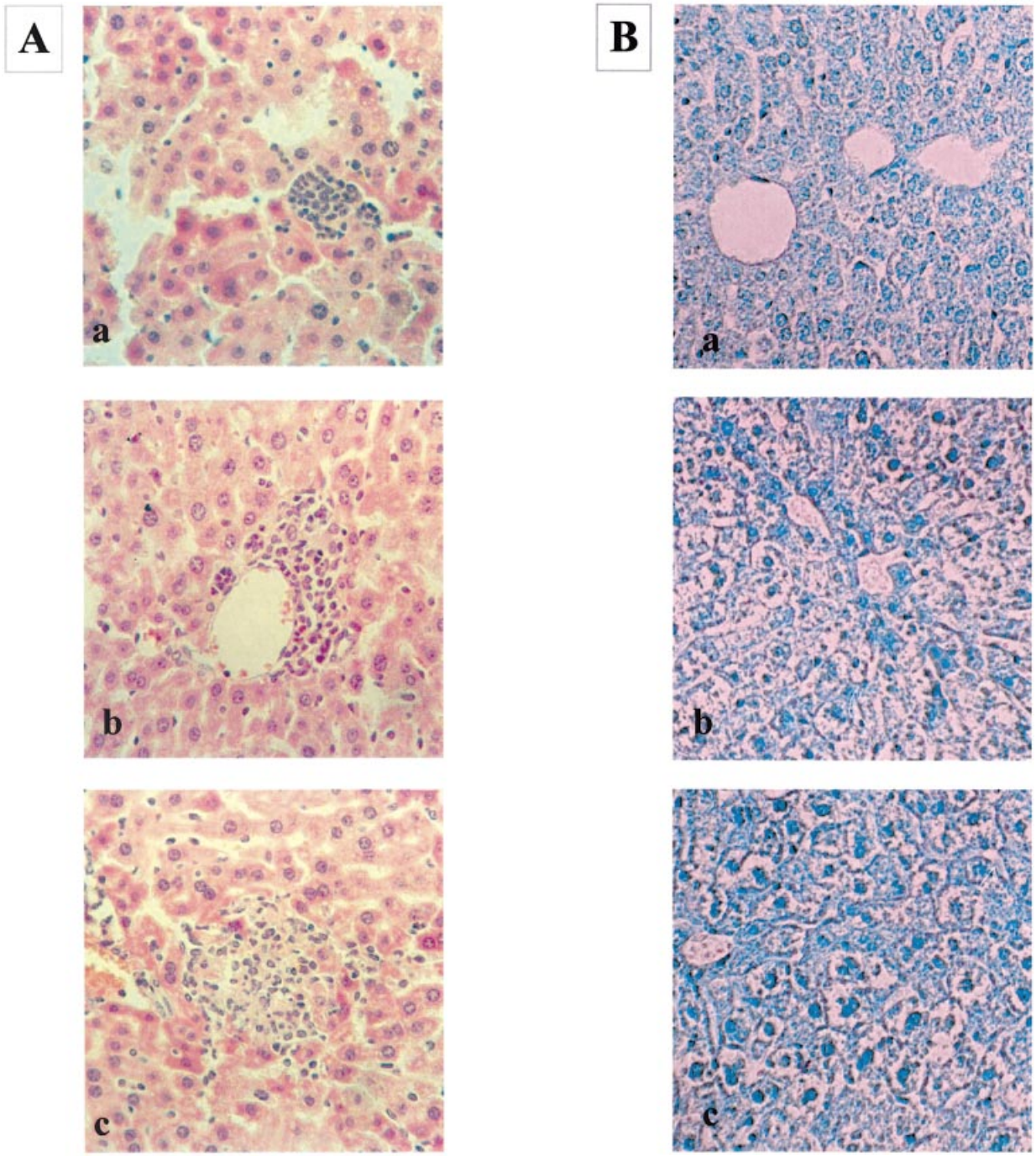

Fig. 6. (A) Livers removed from mice immunized with 23mGST (a–c). (B) Livers from non-immunized mice (a) or from mice immunized with GST23 (b and c). All mice were boosted with the same protein as described in Methods; the tissue slices were stained with hematoxylin & eosin.

mGST23 is a less immunogenic molecule was confirmed. Figure 4 shows the reactivity of antisera for mice immunized with 23mGST, and boosted respectively with mGST (Fig. 4A), fd23 (Fig. 4B) and 23mGST (Fig. 4C).

The panels show that one injection of the chimeric antigen 23mGST is sufficient to break B cell tolerance against mGST self-determinants. Furthermore, a boost with mGST appears to re-stimulate anti-mGST antibody production, thus indicating that once reactivity is established the presence of foreign peptide is no longer needed; conversely the presence of the pep23 sequence is required for re-stimulation of anti-pep23 antibodies (Fig. 4B and C).

T cell proliferation

To determine whether the autoantibody responses are mediated by T cell help specific for mGST self-determinants, we

tested the ability of the recombinant mGST protein to re-stimulate T cells from the spleen or lymph nodes of immunized BALB/c mice. In Fig. 5 we show the *in vitro* proliferation response to mGST in splenocytes and lymph node cells of mice primed respectively with 23mGST or mGST23 and re-stimulated twice with mGST (Fig. 5A and C) or with the same protein used during the first boost. The results show that a proliferative dose-dependent response against mGST can also be obtained in the absence of re-stimulation with the foreign epitope. Control T cell proliferative response against the same antigen in non-immunized mice was similar to the background (data not shown). In conclusion, these results suggest that T cell reactivity against the foreign epitope may spread to mGST-specific determinants, thus providing the help acquired to break B cell tolerance.

Effect of immunization with recombinant antigens on mouse liver

Four mice immunized with mGST23 and four mice immunized with 23mGST, according to the schedule used in the experiments of Fig. 3 and responding to the modified self-antigen, were sacrificed, and their livers were collected. As a control of the effect of bacterial contaminants, animals immunized with schistosomal GST expressed in bacteria and prepared under the same conditions were examined. Furthermore, as a control of the effect of pep23, animals immunized with 23GST were likewise analyzed. Coded liver samples were processed by a histologist and anonymously examined by a pathologist. Conclusions of the blinded analysis performed in all livers from immunized mice exhibiting self-reactivity showed the frequent presence of neutrophil infiltrations accumulating in small foci reminiscent of a mild hepatitis-like situation (Fig. 6A, a–c). Livers from non-immunized mice (Fig. 6B, a) or mice immunized with the schistosomal GST23 protein (Fig. 6B, b and c) exhibited only normal parenchyma, but no infiltrations, thus indicating that the phenomenon observed is correlated with active immunization against self-determinants, and with the presence of anti-mGST antibodies and of self-specific T cells. The finding in this case of specific cells infiltrated into liver tissue is in agreement with published evidence that autoreactive T cells are generally only a small component of these infiltrates, although they may play a major regulatory role (20). The general conditions of the animals before killing were good and no signs of viral infections or other diseases, such as loss of weight or hair, was apparent. This observation, in addition to further supporting the notion that the presence of inflammation signs is due to the induction of an autoimmune humoral and cellular response, also indicates that the treatment is not sufficient *per se* to precipitate overt functional organ damage and clinical symptoms.

Discussion

Autoimmunity may be considered as an undesired consequence of the immune response against microbial or viral infection. Animal models provide evidence that infection by a given agent can induce a particular autoimmune disease, although not all proposed associations appear to be as convincing as one may expect (21). In this frame, the presence of an epitope carried by a microbe and mimicking a self-determinant has been invoked as the explanation for the initiation of autoreactive events (22).

A definition of autoimmune disease may include the presence of autoantibodies as in Grave's disease (hyperthyroidism due to stimulating receptor, myasthenia gravis, pemphigus vulgaris, lupus, immunocytopenia, etc.) (22). In most cases, however, the mechanistic role of autoantibodies in the initiation or maintenance of the autoreaction process is still unclear and, sometimes, production of autoantibodies may simply reflect a secondary event with no or little pathogenetic relevance (22). In recent years, models [including liver (23,24)] are emerging by which disease results not only from breakage of tolerance, leading to generation of an anti-self repertoire, but from the breakdown of additional control systems which should normally keep autoreactive effector cells in check (25). Transgenic mice models suggest the existence of at least two checkpoints, one controlling the activation of autoreactive B and T cells and the spread of recognition of the initial cross-reactive determinant to self-determinants, and another determining the transition from autoreactivity to overt disease (25). For example, breaking peripheral cytotoxic T lymphocyte tolerance in a transgenic mice system fails to induce organ-specific autoimmunity unless a second event occurs, such as infection of the organ by a pathogen (26).

To demonstrate whether cells of the humoral and cellular arms of the immune system, elicited by an infection and cross-reactive to self epitopes, are sufficient to precipitate the clinical symptoms of autoimmunity in mice, animals have been induced by a variety of means to express self-reacting B and T_h cells in the absence of the infecting organism. In addition to transgenic animal models, it was shown that in normal animals immunization with conserved foreign cytochrome *c* can also lead to the activation of autoreactive anti-cytochrome *c* antibody-producing B cells and, coincidentally, to activation of T cell clones directed against self cytochrome *c* (10). Breakage of B cell tolerance has also been obtained by immunization with a highly conserved protein, i.e. ubiquitin, in which a T cell epitope [ovalbumin $(OVA)_{325–336}$] had been inserted by recombinant DNA technologies in order to provide linked help. Such a chimeric protein elicited a strong autoantibody response against self-ubiquitin. A concurrent response of T cells specific for the OVA determinant and for the self-determinants carried by the ubiquitin moiety was induced (11). The continued presence of the immunogen, however, seemed to be required for the maintenance of the autoreactive state. Similarly, anti-self-autoantibodies and T cells can be stimulated by mice immunization with a hybrid ubiquitin carrying not only a T_h cell epitope, such as a universal T cell epitope from *Mycobacterium tuberculosis*, but also a foreign B cell epitope derived from HIV-1 gp120 (13).

In all instances mentioned above, autoreactivity was triggered against an ubiquitously distributed determinant. Although epitope spreading to self-determinants was described, no effect on organ autoimmunity deriving from these treatments, and no information concerning the initiation and maintenance of an autoreactive response leading somehow to disease, was reported. We have developed here an experimental model analogous to that applied by Dalum *et al*. (12) and Lohnas *et al*. (13), but in which a mGST is used as a self-carrier in place of ubiquitin. The use of GST provides potential

advantages due to the availability of a series of structurally related GST molecules (sharing significant-to-poor sequence identity depending on the organisms of origin) for which there is an extensive knowledge of the three-dimensional structure (16). Most notably, different to ubiquitin and cytochrome *c*, GST can exhibit a distinct tissue specificity of expression, thus allowing us to address organ-directed rather than systemic autoreactivity. In the present case, we have chosen to use a murine αGST protein produced in the liver (15). Murine αGST was engineered so to encompass a heterologous sequence derived from HIV-1 reverse transcriptase, and including both a T_h and a B cell determinant (19), as in the model of Lohans *et al*. (13) Immunization of mice with such a recombinant antigen leads to production of antibodies directed against murine GST determinants and to T_h self-epitope spreading. We also found that the presence of foreign help was no longer required after the initial immunization and that self-reactivity could be boosted by successive exposure to murine GST devoid of heterologous help. No evidence of poor competition of autoreactive B cells for limiting help was observed, different from what was found upon immunization with ubiquitin carrying both foreign T and B epitopes (11), a difference probably attributable to the characteristics of the B cell epitopes used in the two studies.

Furthermore, in mice responding to self-GST after breakage of tolerance with recombinant mGST carrying foreign determinants we observed the presence of a distinct, although not massive, neutrophil infiltration, suggesting the onset of a mild hepatitis-like disease in treated animals. The recruitment of polymorphonuclear leukocytes may in fact be important in clearing an infection or disposing of cellular debris, but may also produce host damage (27). Neutrophil infiltration has been reported, for example, in liver during lipopolysaccharide (LPS)-induced inflammation (27). In our case, infiltrations seem to be related directly to the immunizing potential of the antigen rather than to endotoxic shock, because, on the one hand, we tested the absence of LPS in the murine GST preparations used and, on the other hand, schistosomal, non-self-GST, prepared and administered under the same conditions, was unable to trigger both antigen-specific autoreactivity and neutrophil infiltration. Treated mice, however, did not progress further and their health conditions remained normal even after repeated immunization with self-antigens and prolonged stabulation in good practice conditions, suggesting that other events are required to move to the next checkpoint in the progression of the autoimmune disease (7,25), related perhaps to pathogen infections or to other environmental factors. However, although treated animals were monitored for >6–8 months, it should be considered that the clinical signs of autoimmunity may follow the appearance of antibodies by a considerable length of time, thus suggesting that observation of immunized mice should be prolonged to the entire lifespan of the animals. Studies by Matesic *et al*. (27) also suggest that the genetic predisposition to the degree of polymorphonuclear leukocyte infiltration response may play an important role in controlling disease progression. Thus, induction of organ-specific autoreactivity by means of immunization with chimeric self-GST may constitute a useful model for the understanding of liver autoimmune disease and for the identification of precipitating environmental or genetic factors.

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Abbreviations

APC	antigen-presenting cell
CFA	complete Freund's adjuvant
GST	glutathione-S-transferase
IFA	incomplete Freund's adjuvant
LPS	lipopolysaccharide
mGST	murine GST
OVA	ovalbumin

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