Potentiation of immunological tolerance induction in adult mice by co-administration of pooled normal IgG and oral tolerogens: a potential therapeutic approach for autoimmune diseases

José Mengel*, Patrícia Fávaro, André Meyer, Vinícius Motta, Raquel de Alencar, Edilberto Postół, Fabíola Cardillo

Department of Immunology, Institute for Biomedical Sciences IV, University of São Paulo, Av. Prof. Lineu Prestes 1730, CEP 05508-900, São Paulo

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Summary Oral tolerance can be defined as the inability of an adult animal to produce specific antibodies or cellular immune responses upon conventional immunization, after oral antigenic administration. Recently, the oral administration of antigens has gained renewed interest because of the possibility of inducing tolerance in nonimmunized adult animals and, consequently, opening up the theoretical possibility of preventing or treating diseases caused by malfunction of the immune system. This strategy has been proven to be useful in the prevention of allergic and autoimmune diseases in rodents, as well as in the amelioration of certain autoimmune diseases in humans. Although there is experimental and clinical evidence for the usefulness of oral tolerance in medical practice, the mechanisms responsible for this phenomenon are still poorly understood, and the results obtained are not always satisfactory. Herein, we show that the thymus is required for the induction and maintenance of oral tolerance, providing evidence that it is not a pure form of clonal deletion-based peripheral tolerance. Oral tolerance could therefore depend on the formation and release to the periphery of regulatory T cells, such as γδ or αβ T cells, by the thymus. This finding may have profound implications for the treatment of autoimmune diseases, since most of them are associated with thymic hypofunction. On the other hand, due to so far unknown mechanisms, the intraperitoneal co-administration of normal IgG to mice orally treated with tolerogen leads to a sustained and intense immunological tolerance, both in euthymic and thymectomized mice, including those of the lupus erythematosus-prone NZB × NZW lineage. This approach for inducing and maintaining tolerance in thymus-deficient conditions is discussed and put forth herein as a new evidence-based proposition for the therapy of autoimmune diseases.

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* Corresponding author. Tel./fax: +55 713560101x270. E-mail address: jomengel@cpqgm.fiocruz.br, jomengel@terra.com.br (José Mengel).

1 Present address: Oswaldo Cruz Foundation, Gonçalo Moniz Research Center, R. Waldemar Falcão 121, CEP 40295-001 Salvador, Bahia, Brazil. Cellular Immunology, Autoimmunity and Experimental Chagas Disease Laboratory.

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Oral tolerance and autoimmune diseases

The oral administration of antigens has been used to ameliorate autoimmune disorders in rodents and humans in order to re-establish tolerance to self components [1]. Clinical trials have shown that human autoimmune diseases such as multiple sclerosis, uveitis, and arthritis can be treated with relative success, using this approach [2]. Most importantly, such trials reveal the absence of major side effects [1]. However, the overall results of many clinical trials have not been as promising as originally predicted [3]. The discrepancy between the excellent results in the prevention of autoimmune diseases in animal models and the treatment of human autoimmune pathologies probably reflects the influence of several factors, including those discussed below:

(a) Contrasting to the experimental induction of oral tolerance, which is usually carried out in normal animals, in the treatment of autoimmune pathologies in humans it is attempted to re-establish tolerance when the immune system is already committed to an autoimmune state and/or is otherwise malfunctioning [4].

(b) The schedule of antigen administration and doses are well established for rodents, but not for humans [1].

(c) The mechanisms by which oral tolerance is induced and maintained in humans are still unclear [1].

(d) The age of the subject at the time of tolerogenic treatment [5] may be comparatively higher in human patients than in experimental animals.

(e) The nature of the antigens used to induce oral tolerance in experimental animals and human patients may differ [6].

In addition, autoimmune diseases may have distinct pathological mechanisms, and many of them may not be suitable to treatment by the induction of oral tolerance.

Currently, the oral administration of antigens is a promising way of treating autoimmune diseases. However, new insights into the mechanisms responsible for oral tolerization and the development of new protocols or the association of protocols are required so as to improve the clinical results.

In this article, we will discuss the development of the immune system, the interplay among lymphocytes and the results that show an unexpected role of the thymus in the acquisition and maintenance of oral tolerance, through the release of special thymic emigrant cells. Finally, we propose an association of two protocols for the treatment of autoimmune diseases.

T Lymphocyte deletion

Clonally distributed receptors known as T cell receptors (TCRs) and immunoglobulins (Igs) are expressed on T and B cells, respectively [7]. TCRs and Igs are essential for the development of mature T and B cells since the lack of their expression results in the absence of mature T or B lymphocytes [8]. The expression of MHC molecules on thymic epithelial cells is also crucial for the maturation/differentiation of T cells since animals unable to express MHC molecules do not develop mature T lymphocytes [9]. Thus the expression of TCRs and of MHC molecules on thymic stromal cells are required for the appearance of mature T cells. The TCR recognizes peptides bound to MHC molecules [10]. A strong interaction between an immature T cell and a MHC-peptide-presenting cell in the thymus results in the death of that particular T cell with a given specificity (negative selection). A weak interaction rescues immature T cells from apoptosis and sustains the progression of the maturation/differentiation process of a particular T cell that leaves the thymus to peripheral lymphoid tissues (positive selection). A third situation and, probably the most frequent, involves a T cell carrying a particular rearranged TCR which prevents any possible interaction with the array of MHC-peptides expressed by the thymic stromal cells at a given time. In such a case, the thymocytes perish by neglect [11]. The end result of the selection process is the shaping of a T cell repertoire by the elimination of T cells that have high or no avidity for a given combination of MHC-peptide-presenting cells in the thymus. This phenomenon enhances the maturation/differentiation of peptide-MHC-interactive T cells with a low/intermediate avidity for MHC-peptide-expressing thymic cells [11]. This model implies that every mature T cell in peripheral lymphoid organs was positively selected in the thymus by a given array of MHC-peptides (probably of self nature) in the context of a given MHC haplotype [12]. Positive selection is an intricate process [13] in that a given TCR may be positively selected by a balance of a given array of quantitatively or qualitatively different peptides or a given peptide may positively select a
repertoire of certain complexity [14]. These observations suggest that most peripheral T cells are autoreactive to some extent, thus implicating autoreactivity in the existence and survival of T cells in peripheral lymphoid organs [15]. This conclusion does not necessarily imply the existence of suppressor and/or regulatory T cells, as any potentially deleterious autoimmune responses could still be controlled by increasing the ratio of the peripheral negative selection of autoreactive T cells. This would be achieved by increasing the overall avidity between T cells and antigen-presenting cells under microenvironmental conditions with supra optimal antigen presentation [13]. A high concentration of self or non-self antigens would, therefore, cause central and peripheral deletion of T cell-reactive clones, thus resulting in tolerance [16]. Indeed, there is evidence that the oral administration of high concentrations of antigens may lead to partial deletion of responder T cells [17]. However, we show herein that the transfer of splenic cells from orally tolerant BALB/c mice to athymic BALB/c nu/nu recipient mice produces a transient state of tolerance that can be interrupted by successive antigenic boosters. The period required for the recipient mice to recover the immune response varied from 30 days (donor mice tolerantized with low doses of antigens) to 5 months (donor mice tolerantized with high doses of antigen) after transfer. Control euthymic mice that were made tolerant by gavage with high- or low-doses of ovalbumin (OVA) remained tolerant for at least 7 months (Fig. 1). These findings argue against the hypothesis that clonal deletion is the sole mechanism responsible for oral tolerance. They also suggest that high doses of antigens could determine a more stable state of oral tolerance.

### The thymus is required for the establishment of oral tolerance

The long duration of oral tolerance seen in euthymic mice is particularly interesting since thymic emigrant T cells are continuously released from the murine thymus at a rate of approximately one million cells per day [18]. Since these new T cells have not undergone tolerization, it would be reasonable to assume that they would produce normal immune responses upon conventional immunization. Yet, in orally tolerant mice, tolerance persists, as shown herein, for at least 7 months after gavage, a period in which about 200 million new T cells were introduced in the periphery. This observation suggests that other mechanisms, besides clonal deletion, are operating to maintain the state of oral tolerance.

To evaluate the role of the thymus in the induction and maintenance of oral tolerance, one experiment was performed. Fig. 2 shows that DBA/2 mice thymectomized 2 months before gavage with high doses of OVA developed tolerance at the level of specific antibody production. However, unlike euthymic mice, and similarly to athymic nu/nu mice, thymectomized mice were unable to sustain oral tolerance for a long period of time. In another series of experiments, thymectomized F1 (C57Bl/6 × BALB/c) mice underwent gavage with high (three gavages with 5 mg/gavage) or low doses (three gavages with 0.5 mg/gavage) of OVA.
Control imunized mice in these experiments produced $3.1 \pm 0.5$ mg/ml of anti-OVA specific antibodies in the secondary immune response. Mice in which oral tolerance was induced with a low dose of OVA were poorly tolerized ($1.9 \pm 0.4$ mg/ml of anti-OVA specific antibodies in the secondary immune response) compared to those tolerized with a high OVA dose ($0.5 \pm 0.1$ mg/ml of anti-OVA specific antibodies in the secondary immune response). Tolerance in both groups was not sustained for longer than 5 months (data not shown). These results suggest that the thymus is an important organ either in the induction (low antigenic doses) or in the maintenance (low and high antigenic doses) of oral tolerance.

Recent studies have suggested that the thymus may have a major role in the normal immune system functioning in adult mammals. Thus normal thymic activity may maintain an output of "virgin" T cells as well as minor T cell subpopulations with important functional activities in adults. In this case, the thymus serves as an important source of regulatory T cells in the early and late phases of ontogeny [19]. It has been seen that CD4$^{-}$CD45RB$^{high}$, CD4$^{+}$CD25$, NK1.1^{+}$αβ$^{-}$ and γδ$^{-}$ T cells, which are among thymic emigrant T cells, have regulatory functions either in the generation of memory or in the induction of tolerance [20–25].

### γδ T cells as regulatory cells in the induction and maintenance of oral tolerance

γδ T cells show distinct functional patterns and are involved either in the induction and maintenance of tolerance or in the upregulation of immune responses. αβ$^{+}$ CD4$^{+}$ T cells from γδ T cell-depleted mice show increased levels of IL-2 production and thymidine incorporation when cultured with syngeneic filler cells [27]. Hepatic γδ T cells from mice made tolerant by the transfer of spleen cells from minor histocompatibility-mismatched mice induced tolerance in naïve recipients [27]. γδ T cells can also down-regulate the helper activity of αβ$^{+}$ CD4$^{+}$ T cells by suppressing IgE production in vivo [28]. Splenic γδ T cells from Trypanosoma cruzi-infected mice can block mixed lymphocyte reaction proliferation and the production of IFN-γ by αβ$^{+} T$ cells in vitro [29]. The latter γδ T cell suppressor activity was recently shown to be thymus dependent [24]. In this model, non-specific suppression of the antibody response could be reversed by in vivo γδ T-cell depletion. Furthermore, we have demonstrated that γδ T cell-depleted mice are not susceptible to the induction of oral tolerance with OVA and that, once established, oral tolerance could be terminated by depleting γδ T cells [30]. The latter results have been confirmed and extended in δ knockout mice [31]. In addition, insulin inhalation can delay or prevent diabetes in NOD mice, an effect mediated by γδ T cells [32]. Also, a positive regulatory influence of γδ T cells on αβ T cells has also been demonstrated [33].

γδ T cells of thymic origin are short-lived [34] and consist of CD3$, CD28$, CD40-ligand$, Fas$^{-}$, FcyR$^{-}$ cells [35]. In fact, we have detected low percentages of γδ$^{-}$ Fas$^{+}$ cells after thymectomy ($6 \pm 2.8\%$ of Fas$^{-}$ γδ$^{-}$ T cells among splenic γδ$^{-}$ T cells) and an increase in their levels after the injection of thymocytes into thymectomized mice ($14.8 \pm 4.7\%$ of Fas$^{-}$ γδ$^{-}$ T cells among splenic γδ$^{+}$ T cells). In addition, it was recently described that a subpopulation of Vγ1$^{-}$ γδ T cells are able to kill activated macrophages via Fas–FasL interaction [36]. This study is of particular interest as it may contribute to the understanding of the mechanism by which a subset of γδ T cells could control the
activation of conventional $\alpha\beta^+$ T cells, albeit in an indirect way by diminishing the numbers of potential activated antigen-presenting cells. Other studies support the idea that $\gamma\delta$ T cells have, or acquire, different effector functions, depending on their interactions with the microenvironment [37]. The studies and results discussed herein strongly suggest the participation of the thymus in the induction and maintenance of oral tolerance in normal animals through positive selection of one or more T cell lineages.

Immunoglobulins and oral tolerance

Although B cells are not fundamental for the induction of tolerance [38], they may be important in the regulation of immune responses. For instance, B cells may work as regulatory cells in certain experimental autoimmune diseases [39] or in the maintenance of T cell anergy [40]. The mechanism by which B cells exert these effects is not yet understood. However, a recent report has shown that animals which do not express the Fc gamma receptor because of a gene target mutation are highly susceptible to the induction of arthritis upon collagen immunization [41]. These results indicate that Fc receptors and immunoglobulins are involved in the regulation of immune responses, as previously suggested [42]. Indeed, the administration of large quantities of human immunoglobulins to patients with different types of autoimmune disease has been shown to improve their clinical condition [43]. Thus B cells, through their major product (immunoglobulins), could theoretically be important in the regulation of oral tolerance. In fact, euthymic F1(C57Bl/6 × BALB/c) mice intraperitoneally (i.p.) injected with protein-A purified IgG from pooled normal mouse sera and subjected to oral tolerization with low doses of antigen become highly tolerant, indicating a potentiation of the classical protocol by the administration of normal immunoglobulins. The same type of experiment was done in young F1(NZB × NZW) mice. These mice are totally resistant to the induction oral tolerance by the use of relatively low doses of antigen. However, the administration of pooled normal IgG (i.p.) and antigen by the oral route renders NZB × NZW mice completely tolerant at the level of the specific humoral response (Fig. 3). These results strongly suggest that the co-administration of pooled normal IgG potentiates the induction of oral tolerance in normal or autoimmune prone mice strains.

Figure 3 Purified normal mouse IgG potentiates the induction of oral tolerance in mice. Two-month-old F1(C57Bl/6 × BALB/c) or F1(NZB × NZW) mice were either injected i.p. with 1 mg of protein-A purified normal IgG from pooled sera or PBS every other day, starting 1 week before the initial gavage procedure and continuing for up to 15 days post-gavage (days $-7$, $-5$, $-3$, $-1$, +1, +3, +5, +7, +9, +11, +13, +15). The mice also underwent to three gavages with PBS or 0.5 mg or 2 mg of OVA for F1(C57Bl/6 × BALB/c)/F1(NZB × NZW) mice respectively, on days 0, +1 and +2. Mice were then immunized (i.p.) with 100 $\mu$g of OVA in alum (1 mg/animal) 7 days after the initial gavage and with 100 $\mu$g of soluble OVA on day +14. Sera were collected 14 days after the secondary immunization and specific antibodies to OVA quantified by ELISA (see Fig. 1). The columns represent the mean ± SD ($n = 4–7$ mice).

Lymphopenia and autoimmune diseases

Autoimmune diseases are in general associated to T-cell lymphopenia [44]. Lymphopenia may be due to deficient thymic function and/or peripheral T cell loss due to terminal differentiation, recruitment to inflammatory sites and death [45,46]. In addition, in many autoimmune diseases the thymic function is severely reduced, both in rodents and in human beings [47–50]. In fact, thymic hypofunction might be one of the causes of autoimmune diseases [44]. More importantly, as we discussed above, a functioning thymus is required for in the induction and/or maintenance of oral tolerance in normal animals. These observations might account for the modest results found in many clinical trials that use oral tolerance as a principle to reestablish self-tolerance. Therefore, therapies aiming at treating autoimmune diseases might consider the correction of T cell lymphopenia by means of restoring
thymic function (production and/or export of regulatory T cells) or, alternatively, to be based in strategies for inducing peripheral tolerance that do not require normal thymic function. In Fig. 4 we show that either thymectomized F1(BALB/c × C57Bl/6) or autoimmune prone thymectomized F1 (NZB × NZW) could be rendered extremely tolerant at the level of antibody production to low doses of antigen (OVA), administered by the oral route when polyclonal IgG was given i.p., concomitantly. Also, these thymectomized animals remained tolerant for more than 6 months (data not shown). These experiments show that the combination of the oral tolerance protocol with the co-administration of normal pooled IgG bypasses the requirement for a functional thymus and therefore, might be of extremely importance in the treatment of some autoimmune diseases.

Concluding remarks

1. A functioning thymus seems to be crucial for the induction and/or maintenance of oral tolerance by exporting regulatory/suppressor cells in normal animals.
2. Thymic function is decreased in autoimmune diseases.
3. The induction of oral tolerance in normal or autoimmune prone strains of mice is extremely potentiated by the co-administration of pooled IgG from normal mice.
4. Oral tolerance to low doses of antigen can be induced and maintained in thymus-less animals, having normal or autoimmune genetic backgrounds, that received normal IgG. Therefore, the mechanism underlying this biological effect might be, purely, peripheral.
5. Treatments aiming the amelioration of autoimmune diseases could combine both protocols (oral tolerance and intravenously administration of Ig (IV-Ig) as it may be more effective, without important predictable side effects.

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