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Intravenously administered contact allergens coupled to syngeneic erythrocytes induce in mice tolerance rather than effector immune response

Katarzyna Nazimek[1,2], Magdalena Wąsik[1], Philip W. Askenase[2], Krzysztof Bryniarski[1,2]

[1]Department of Immunology, Jagiellonian University Medical College, Kraków, Poland
[2]Section of Rheumatology, Allergy and Immunology, Department of Internal Medicine Yale University School of Medicine, New Haven, USA

Corresponding author: Krzysztof Bryniarski, PhD, Prof.
Department of Immunology, Jagiellonian University Medical College
ul. Czysta 18, 31-121 Kraków, Poland
Phone/Fax: +48 12 633 94 31; E-mail: mmbrynia@cyf-kr.edu.pl

Abstract: Constantly increasing prevalence of allergic diseases determines the attempts to elaborate the therapeutic strategies activating immune tolerance to particular allergen. Our current research focuses on the antigen-specific action of CD8+ suppressor T (Ts) lymphocytes induced in mice by intravenous administration of a high dose of haptenated syngeneic erythrocytes. While the regulatory activity of Ts cells mediated by exosome-delivered miRNA-150 is well defined, the mechanism of their induction remained unclear. Therefore, the current studies investigated the immune effects induced in mice by intravenous administration of contact allergens coupled to syngeneic erythrocytes. In mouse models of hapten-induced contact hypersensitivity (CHS) and delayed-type hypersensitivity to ovalbumin, we have shown that intravenous administration of hapten-coupled erythrocytes failed to induce CHS effector cells. Moreover, hapten-induced CHS reaction occurred to be suppressed in mice intravenously administered with syngeneic erythrocytes coupled with protein allergen. Finally, we have demonstrated that intravenously administered allergen induces immune tolerance only when bound to syngeneic erythrocytes, proving that intravenously delivered allergens are deprived of their immunizing properties when coupled with membrane of self cells. Altogether, our current studies suggest that alteration of self cell membrane by allergen binding is enough to induce Ts cell-mediated immune tolerance to nonpathogenic agents, which express a great translational potential in such conditions as allergies and hypersensitivity-related autoimmune disorders.

Key words: allergen, allergy, contact hypersensitivity, delayed-type hypersensitivity, immune suppression, immune tolerance, sensitization, suppressor T cells, tolerogen.

Introduction

The increasing prevalence of allergies and hypersensitivities, including contact allergy, became at present one of the most recognizable adverse effects of economic development of societies and environmental pollution to human health. As estimated, more than 20% of people of the general population suffer from contact allergy [1], which is associated with an increased frequency of exposure to common allergens present in house and workplace environment, in food, drugs and other consumer goods. While generally available and most commonly used means of treatment of allergic patients usually induce the state of antigen-non-specific immune suppression, current studies attempt to elaborate the therapeutic strategies activating immune tolerance to particular allergen, and remaining the responsiveness of immune cells to other antigens unaffected.

While haptenated proteins possess immunostimulatory and allergy-inducing properties [2], haptens bound to membrane structures of self cells were suggested to act as tolerogen [3]. Along these lines, our current research focuses on the action of CD8+ suppressor T (Ts) lymphocytes induced in mouse model of contact hypersensitivity (CHS), a cutaneous manifestation of delayed-type hypersensitivity (DTH) that reflects human contact dermatitis [4], by intravenous administration of a high dose of haptenated syngeneic erythrocytes [5–8]. Ts cells have been already proved to suppress hapten-induced CHS reaction in a hapten-specific manner by the release of miRNA-150 contained in exosomes coated with hapten-specific antibody free light chains (LCs) [5], ensuring the specificity of induced suppressive effect [7]. Similarly, our recent research findings showed that exosome-carried miRNA-150 also antigen-specifically down-regulates mouse DTH response to food allergens, such as ovalbumin (OVA) and casein [9, 10]. Exosome-carried miRNA-150 target antigen presenting cells, likely by binding antigenic determinants complexed with surface MHC molecules via LCs of corresponding specificity [11], which in turn inhibit effector T cells of immune response [8, 12]. Thus, due to the generation of suppressive exosomes, Ts cells are able to activate long-lasting immune tolerance to particular antigen determined by the specificity of LCs coating exosomes and produced by B1 cells at the time of sensitization with antigen [6, 7, 13]. This mechanism seems to have a great translational potential in allergen-specific therapy of contact allergy. However, while the regulatory activity of Ts cell-derived exosomes delivering miRNA-150 is well defined [5–12], the mechanism of induction of Ts cells by intravenously administered, hapten-coupled syngeneic erythrocytes remains still weakly understood. Therefore, the current studies have been undertaken to investigate the immune effects induced in mice by intravenous administration of DTH-eliciting allergens coupled to syngeneic erythrocytes.

Materials and Methods

Nine to twelve week-old CBA mice either from the Breeding Unit of Faculty of Medicine of the Jagiellonian University Medical College (Krakow, Poland) or from Jackson Laboratories (Bar Harbor, ME) were subjected to experiments according to the guidelines of the 1st Local Ethics Committee (approval number 39/2011, 40/2011 and 106/2012) and the Institutional Animal Care and Use Committee of the Yale University, New Haven, CT (Permit Number 07381). Each experiment was repeated at least twice and representative results were interpreted and depicted in the figures.

Freshly collected mouse syngeneic erythrocytes were conjugated with trinitrophenyl (TNP) hapten by resuspending of 1 ml of erythrocyte pellet in 7 ml of trinitrobenzene sulphonic acid (Eastman Chemicals, Rochester, NY) solution in cacodylic buffer (5.7 mg/ml), and incubating 20 minutes at room temperature on hematological roller and in darkness. Similarly, erythrocytes in 10 ml of 10% DPBS suspension were coupled with oxazolone (OX) hapten (Sigma, St Louis, MO) by mixing with 20 ml of OX solution, prepared earlier by dissolving 10 mg of OX sample in 1 ml of 96% ethanol and then in 20 ml of boiling DPBS, and incubating for 10 minutes in analogous conditions. Finally, erythrocytes were labelled with protein antigens, i.e. OVA or bovine serum albumin (BSA), both from (Sigma, St Louis, MO), by modified method of Jandl and Simmons [14]. Briefly, 1 ml of 50% DPBS suspension of mouse erythrocytes was mixed with 5 ml of 1% OVA or BSA solution in DPBS containing Cr^{3+} ions from 2.5 micromolar solution of $CrCl_3$, and incubated for 1 hour in the same conditions. Where indicated, erythrocytes were incubated only in the presence of Cr^{3+} ions, in the corresponding volume of DPBS. After washing, resuspending in DPBS and filtration through nylon mesh, erythrocytes were used for intravenous administration as allergen-coupled mouse syngeneic erythrocytes (MRBC), i.e. TNP-MRBC, OX-MRBC, OVA-MRBC or BSA-MRBC, respectively, or as chromium-treated erythrocytes (Cr^{3+}/MRBC).

Mice were injected intravenously on day 0 and 4 with 0.2 ml of 10% DPBS suspension of allergen-coupled MRBC or chromium-treated MRBC, or, where indicated, with 1 mg of OVA in 0.2 ml of DPBS. Three days later mice were either contact sensitized by topical application of 0.15 ml of 5% trinitrophenyl chloride (picryl chloride, PCL, Chemtronix, Swannanoa, NC) or 3% OX solution in a mixture of ethanol and acetone (3:1 *v/v*) on shaved abdominal skin [5–8], or immunized by 4 intradermal injections of 0.05 ml of 0.5 mg/ml OVA solution in physiological saline on 2 consecutive days [15]. In some instances, recipients of TNP-MRBC remained not sensitized prior to ear challenge. After 5 days mice were ear challenged to elicit CHS or DTH responses, respectively, by topical application on each side of both ears of 10 μl of 0.4% PCL or OX in a mixture of acetone and olive oil (1:1 *v/v*), or by

intradermal injections into both ears of 10 µl of a 0.5 mg/ml OVA solution in saline. Twenty four hours later ear swelling was measured with an engineer's micrometer (Mitutoyo, Japan) by an observer unaware of experimental protocol [16]. Background ear thickness was then subtracted to calculate ear thickness increase for each mouse. Afterwards, nonspecific increase in thickness of ears of non-sensitized but challenged littermates, caused by chemical irritation of skin by antigen and its vehicle, in most cases was subtracted from experimental groups to calculate a net value of ear swelling expressed as *delta* ± standard error (SE) [U × 10^{-2} mm]. Statistical significance of the differences observed between groups was estimated, when test assumptions were accomplished, in Analysis of Variance (ANOVA) with *post hoc* RIR Tukey test and $p < 0.05$ was considered statistically significant.

Results

Intravenous administration of hapten-coupled erythrocytes failed to induce CHS effector cells

Previous research findings unequivocally proved that intravenous administration of hapten-coupled syngeneic erythrocytes prior to contact sensitization with the same hapten leads to the suppression of subsequently elicited CHS reaction [3] by activation of Ts cells releasing regulatory miRNA-150 in exosomes [5–12]. However, it was undefined whether intravenously delivered hapten-coupled erythrocytes may also induce CHS effector cells, similarly to haptenated proteins [2]. To test this assumption, recipients of TNP-MRBC were remained not sensitized prior to ear challenge with TNP hapten that presumably should elicit CHS ear swelling. Notably, the resulting increase in ear thickness of TNP-MRBC-administered mice was comparable to this measured in similarly challenged, non-sensitized littermate animals, which results only from chemical irritation of skin by hapten solution, while the ear thickness increase caused by inflammatory reaction of CHS effector cells in hapten-sensitized mice was significantly higher (Fig. 1, Groups C and A vs B). This observation undoubtedly proves that intravenous administration of erythrocytes coupled with hapten does not induce CHS effector cells. Instead, this mean of treatment leads to induction of immune tolerance mediated by Ts cells.

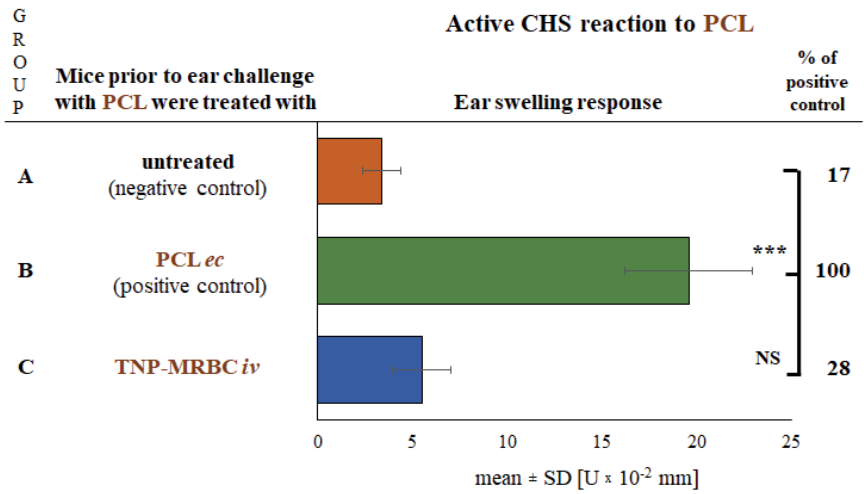


Fig. 1. Hapten-coupled syngeneic mouse red blood cells (MRBC) failed to induce contact hypersensitivity (CHS) effector cells after intravenous (*iv*) administration. Mice were injected intravenously on day 0 and 4 with 0.2 ml of 10% DPBS suspension of trinitrophenol (TNP)-coupled MRBC. On day 7 mice of positive control group were epicutaneously (*ec*) sensitized with 0.15 ml of 5% trinitrophenyl chloride (picryl chloride, PCL) solution. Then, on day 12 mice of all groups were ear challenged with 0.01 ml of 0.4% PCL solution to elicit CHS ear swelling, measured 24 hours later with an engineer's micrometer. Results are shown as mean increase of ear thickness [U × 10^{-2} mm] ± standard deviation (SD). One-way ANOVA with *post hoc* RIR Tukey's test; *** p <0.005.

Hapten-induced CHS reaction is suppressed in mice intravenously administered with syngeneic erythrocytes coupled with protein allergen

Allergy to red meat with immediate and delayed onsets is observed in humans [17], and BSA is considered as one of potential allergens. Furthermore, methylated BSA was recently shown to induce DTH response in mice [18]. Conversely, we have recently found that suppression of TNP-induced CHS reaction could be achieved by intravenous administration of syngeneic erythrocytes coupled with protein antigen, namely BSA. It was assumed that the specificity of induced suppression depends on the hapten used for further epicutaneous sensitization, since it activates B1 cell secretion of specific LCs coating the regulatory exosomes, and that coupling with protein antigen is enough to alter self antigens of syngeneic erythrocytes to make them tolerogenic [7]. However, it was unclear whether this phenomenon generally applies to hapten-induced CHS response or depends on hapten used for subsequent sensitization. To verify these doubts, we have intravenously administered mice with BSA-MRBC prior to contact sensitization with OX hapten, that is non-cross reactive with TNP [3, 5, 6]. Accordingly, CHS ear swelling reaction elicited by challenge with OX was similarly suppressed in both, mice tolerized with OX-MRBC and BSA-MRBC

(Fig. 2, Groups B and C vs A). This strongly suggested that alteration of syngeneic erythrocytes by binding of protein allergens is also efficient to induce Ts cells suppressing CHS response to various haptens, when such tolerogen is delivered via intravenous route.

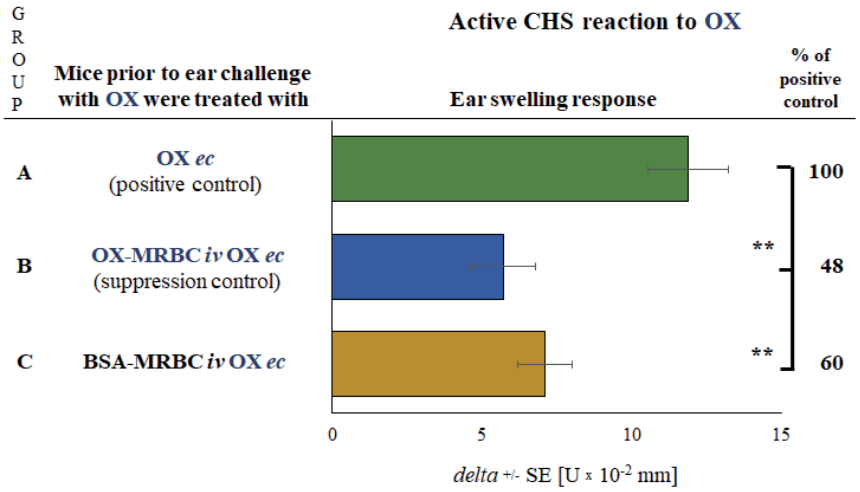
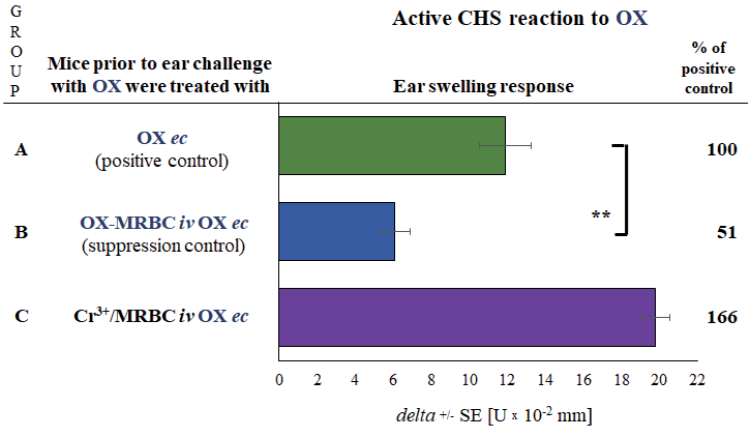


Fig. 2. Syngeneic mouse red blood cells (MRBC) coupled with bovine serum albumin (BSA) suppress oxazolone (OX)-induced contact hypersensitivity (CHS) response after intravenous (*iv*) administration. Mice were injected intravenously on day 0 and 4 with 0.2 ml of 10% DPBS suspension of either BSA-MRBC or OX-MRBC. Three days later mice were epicutaneously (*ec*) sensitized with 0.15 ml of 3% OX solution, and on day 12 mice were ear challenged with 0.01 ml of 0.4% OX solution to elicit CHS ear swelling, measured 24 hours later with an engineer's micrometer. Results, after subtracting of background increase of ear thickness in non-sensitized but challenged mice, are shown as *delta*; i.e. net value of ear swelling [U × 10^{-2} mm] ± standard error (SE). One-way ANOVA with *post hoc* RIR Tukey's test; ** $p < 0.01$.

However, Cr^{3+} ions were used herein as activator of coupling of proteins to erythrocyte membrane. Since chromium is also a well known contact allergen [19], this rises some doubts regarding the actual agent altering mouse erythrocytes to make them tolerogenic, whether indeed it is a protein or chromium ions. To address these concerns, we had treated mouse syngeneic erythrocytes as for coupling with protein antigen, but without adding it. Then, such chromium-treated erythrocytes were injected intravenously to mice prior to sensitization with PCL or OX hapten, but in both cases it failed to suppress elicited CHS ear swelling (Fig. 3a and 3b, Group C vs B and A). Apparently, efficient tolerization could be achieved by tolerogen composed of the complex allergenic molecule bound to membrane of syngeneic erythrocyte.

a.



b.

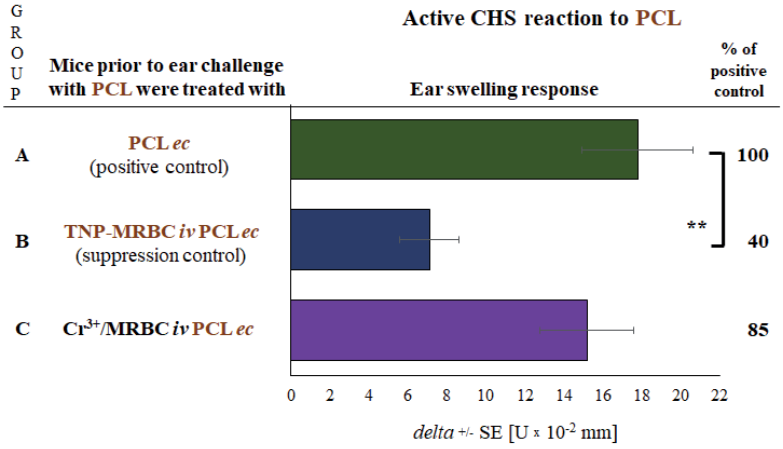


Fig. 3. Chromium ions (Cr^{3+}) failed to make intravenously (*iv*) administered syngeneic mouse red blood cells (MRBC) suppressive in **a.** oxazolone (OX) and **b.** trinitrophenol (TNP)-induced contact hypersensitivity (CHS) response. Mice were injected intravenously on day 0 and 4 with 0.2 ml of 10% DPBS suspension of either Cr^{3+}/MRBC, OX-MRBC or TNP-MRBC. Three days later mice were epicutaneously (*ec*) sensitized with 0.15 ml of either 3% OX solution or 5% trinitrophenyl chloride (picryl chloride, PCL) solution, and on day 12 mice were ear challenged with 0.01 ml of 0.4% OX or PCL solution to elicit CHS ear swelling, measured 24 hours later with an engineer's micrometer. Results, after subtracting of background increase of ear thickness in non-sensitized but challenged mice, are shown as *delta*; i.e. net value of ear swelling [U × 10^{-2} mm] ± standard error (SE). One-way ANOVA with *post hoc* RIR Tukey's test; ** $p < 0.01$.

Intravenously administered allergen induces immune tolerance only when bound to membrane of syngeneic erythrocytes

As mentioned above, the complex allergen molecules bound to cell membrane and administered intravenously efficiently induced immune tolerance. However, it still remained unclear whether coupling of allergen to cell membrane is absolutely essential. To resolve this issue, we have chosen the OVA-induced DTH model [15]. Firstly, because we have initially found that DTH to OVA could also be suppressed by intravenous administration of OVA-coupled MRBC [5–12], and secondly to avoid the uncontrolled binding of intravenously delivered, soluble hapten to membrane proteins of self erythrocytes in mouse recipient circulation [3, 20]. Thus, we have intravenously administered mice with a high dose of OVA solution in DPBS prior to intradermal immunization with OVA, but it failed to affect subsequently elicited DTH ear swelling response, in contrast to OVA-coupled MRBC (Fig. 4, Group B vs C and A). This observation has ultimately proved that binding of allergen to cell membrane prior to intravenous administration is crucial for induction of immune tolerance.

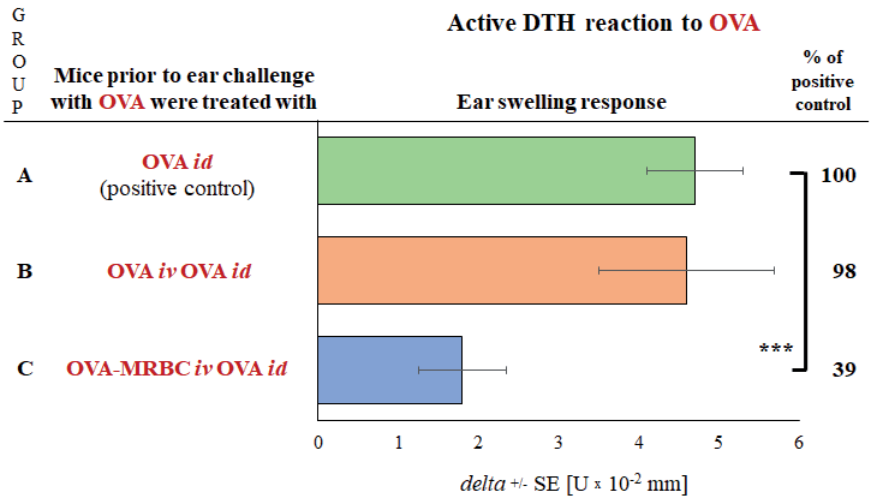


Fig. 4. Intravenously (*iv*) administered high dose of ovalbumin (OVA) suppress OVA-induced delayed-type hypersensitivity (DTH) response only after coupling with syngeneic mouse red blood cells (MRBC). Mice were injected intravenously on day 0 and 4 either with 1 mg of OVA in 0.2 ml of DPBS or with 0.2 ml of 10% DPBS suspension of OVA-MRBC. On days 7 and 8 mice were intradermally (*id*) immunized with total volume of 0.2 ml of 0.5 mg/ml OVA solution in physiological saline, and on day 12 mice were ear challenged with 0.01 ml of 0.5 mg/ml OVA solution in physiological saline to elicit DTH ear swelling, measured 24 hours later with an engineer's micrometer. Results, after subtracting of background increase of ear thickness in non-sensitized but challenged mice, are shown as *delta*; i.e. net value of ear swelling [U × 10^{-2} mm] ± standard error (SE). One-way ANOVA with *post hoc* RIR Tukey's test; *** $p < 0.005$.

Discussion

Depending on the route of its administration, antigen could be either immunogenic or tolerogenic [20]. This observation is important in attempts to induce immune tolerance to chosen antigens. Accordingly, while intradermal immunization of mice with OVA provokes the development of DTH response [15], oral delivery of this protein induces immune tolerance mediated by extracellular vesicles, so-called tolerosomes [21, 22]. Furthermore, oral tolerance to OVA could be induced by feeding mice with modified probiotic bacteria secreting OVA [23]. Along these lines, skin-induced immune suppression could be achieved by epicutaneous application of haptenated protein antigens [24]. Furthermore, epicutaneous immunization with myelin basic protein prior to induction of experimental autoimmune encephalitis occurred to activate regulatory cells preventing the development and ameliorating disease severity [24]. Recently this mechanism was translated into therapy of multiple sclerosis in human beings [25].

Similarly, contact sensitizing haptens, when administered intravenously, were found to impair the reactivity of immune cells [20]. Detailed analysis of this phenomenon suggested that haptens bound to self cell membrane act as tolerogens [3]. Simultaneously, it was shown that intravenously administered, hapten-coupled syngeneic erythrocytes activate in mice Ts cells, down-regulating CHS reaction to this hapten [3, 20]. However, the mechanism of tolerance induction by intravenously administered, hapten-coupled syngeneic erythrocytes remained weakly understood. The current studies investigating the immune effects induced in mice by intravenously administered contact allergens coupled to syngeneic erythrocytes brought two interesting new findings. Firstly, it was proved that intravenously delivered, allergen-coupled syngeneic erythrocytes do not activate the development of DTH effector cells, and secondly that the resulting immune tolerance is induced by complex allergens bound to self cell membrane. On the other hand, it was confirmed in another hapten system that the actual specificity of induced immune tolerance is determined by the sensitizing allergen. Thus, this observation seems to make the studied mechanism of tolerance induction more general than previously concerned.

Along these lines, one can speculate that the tolerogenic effect of allergens bound to membranes of self cells developed in fact as preventive mechanism against immunization to cell membrane proteins altered by such allergens. An indirect evidence that may confirm this speculation is brought by considering the type II allergy, drug-induced cytopenia especially, in which not all of drug-treated patients become sensitized. Instead, those individuals that developed the clinical manifestations of drug-induced allergic reaction are recognized as prone to sensitizing agents. However, it could be hypothesized that their susceptibility to allergens may partly results from breaking of immune tolerance. Drug-induced cytopenia results from

binding of accumulated in plasma drug-derivatives of hapten trait to cell membrane structures, followed by generation of hapten-specific IgG forming immune complexes with haptenated self antigens of blood cells [26]. However, drug-derived haptens likely bind to self blood cells of individuals not developing clinical signs of type II allergy as well. Thus, this may reflect the tolerogenic activity of described herein allergen-coupled self erythrocytes after intravenous administration. Similar tolerogenic effect was also suggested in the case of intravenous administration of a high dose of soluble hapten, shown to rapidly conjugate to circulating blood cells [3]. Altogether, these findings imply that haptens and allergens, when bound to plasma membrane of self cells in the circulation, act as tolerogen, so thus development of allergic reaction is caused by breaking of the induced immune tolerance. Since the effector phase of type II allergy involves cytolysis of haptenated self cells bearing immune complexes on their surface [26], such tolerance mechanism may be considered protective against destruction of self cells altered by nonpathogenic agents, that is observed in autoimmunity.

Immune tolerance induced by intravenously administered syngeneic erythrocytes coupled with allergen was found to be mediated by CD8+ Ts cells, that are lacking the expression of classical regulatory T cell markers, like FoxP3 [5], which suggests that they belong to population of naturally-occurring suppressor T lymphocytes [27]. Those were proposed to be involved in maintenance of immune tolerance to self antigens [28], and to control macrophage immune functions, including phagocytosis and presentation of antigens [29]. CD8+ Ts cells induced by contact allergen-coupled self erythrocytes suppress DTH response to subsequently immunizing allergen by releasing miRNA-150-carrying exosomes coated with hapten-specific LCs ensuring the specificity of immune tolerance [5–12, 20]. Notably, our research findings suggest that alteration of self cell membrane by allergen binding is enough to induce Ts cell-mediated immune tolerance to nonpathogenic agents. This has a great translational potential in such conditions as allergies and hypersensitivity-related autoimmune disorders or immune responses to antigens of transplanted organs.

Induction of tolerant Ts cells by intravenous delivery of a high dose of allergen-coupled erythrocytes to some extent resembles the activation of tolerance after exposure to high doses of allergen, observed in the case of beekeepers and owners of domestic animals, cats especially [30]. Thus, described herein immune tolerance mechanism could presumably be classified as naturally-occurring. On the other hand, our current research findings imply that self blood cells could constitute an allergen-delivery system for utilizing in allergen-specific immunotherapy [30], thereby contributing to inducible mechanisms of immune tolerance. Furthermore, other studies revealed that the type of cell used for coupling with allergen seems not to have a crucial impact on induction of immune tolerance [3]. Consequently, one can speculate that the tolerant state could be induced in each condition associated

with the formation of allergen-self cell membrane conjugates. It is worth noting that currently studied haptens and protein antigens belong to a group of contact allergens. Therefore, described herein mechanism of tolerance induction also applies to DTH-related disorders, including contact dermatitis.

Another important discovery of the present study proves that intravenously administered contact allergens are deprived of their immunizing properties by binding to membranes of self cells, since they failed to induce the maturation of CHS effector cells. This observation additionally supports the aforementioned assumption regarding the possibility of clinical utilization of allergen-self cell membrane conjugates, acting as tolerogen, in allergen-specific immunotherapy. Accordingly, from the clinical point of view, it would also be interesting to investigate whether Ts cell-mediated tolerance could be induced by application of tolerogen via other, less invasive routes, oral especially.

To summarize, our current research contributes to the recognition of recently discovered tolerance mechanism by bringing new knowledge on the process of Ts cell induction by intravenous administration of allergen-coupled syngeneic erythrocytes. We found that intravenously delivered, contact allergen-coupled syngeneic erythrocytes induce tolerant state, instead of DTH effector cells, and that the resulting immune tolerance is induced by complex allergen molecules conjugated to plasma membrane of self cells. Furthermore, we have verified the observation that the specificity of CHS suppression is determined by the sensitizing hapten. Therefore, our current findings seem to have significant clinical implications.

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Disclosures

K.N. planned and performed the experiments, analyzed the results and drafted the manuscript; M.W. assisted in performance of experiments; P.W.A. contributed with reagents and materials, consulted the experimental protocols and revised the manuscript; K.B. planned and performed the experiments, analyzed the results and revised the manuscript.

Conflict of interest

None declared.

Abbreviations

BSA — bovine serum albumin
CHS — contact hypersensitivity
DPBS — Dulbecco's phosphate-buffered saline
DTH — delayed-type hypersensitivity
LCs — light chains
MHC — major histocompatibility complex
MRBC — mouse red blood cells
OVA — ovalbumin
OX — oxazolone
PCL — picryl (trinitrophenyl) chloride
TNP — trinitrophenol
Ts — suppressor T cells

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