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RM-11, an isoxazole derivative, accelerates restoration of the immune function in mice treated with cyclophosphamide

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Abstract:
The aim of this study was to evaluate efficacy of an isoxazole derivative RM11 to accelerate reconstitution of selected immune activities in cyclophosphamide (CP)-immunocompromised mice. We demonstrated that administration of fifteen 10 μg intraperitoneal doses of RM11, following a sublethal (200 μg/kg) dose of CP, significantly stimulated the number of antibody-forming cells (AFC) to sheep erythrocytes (SRBC) as determined 35 days after the CP treatment. Similarly, treatment of the CP-injected mice with 7 doses of RM11 significantly enhanced generation of delayed type hypersensitivity (DTH) to ovalbumin (OVA). Moreover, in that model, the treatment of mice with RM11 accelerated the process of myelopoiesis. RM11 also counteracted the suppressive action of methotrexate (MTX) in the *in vitro* model of the humoral immune response to SRBC. The phenotypic studies with fluorocytometer revealed that intraperitoneal 10 μg dose of RM11 significantly elevated the percentage of mature ($CD3^+$, $CD4^+$ and $CD8^+$) T cells in the spleen and down-regulated the content of $CD19^+$ cells. We conclude that RM11 may be of potential therapeutic value in restoration of the immune status in patients undergoing chemotherapy.

Key words:
isoxazoles, mice, immune response, cyclophosphamide, methotrexate

Abbreviations: AFC – antibody forming cells, CP – cyclophosphamide, DMSO – dimethyl sulfoxide, DTH – delayed type hypersensitivity, MTX – methotrexate, OVA – ovalbumin, PBS – phosphate buffered saline, SRBC – sheep red blood cells

Introduction

Advances of modern medicine increase the demand for new pharmacologically active compounds of both

inhibitory and stimulatory properties in the immune system. The heterocyclic structure of isoxazole has been one of the main sources of drugs introduced to therapy. We described a number of compounds bearing the basic isoxazole structure, which expressed predominantly immunosuppressive properties [10, 15, 17–19, 23]. However, we also described new isoxazole derivatives, amides of 5-amino-3-methyl-4-isoxazolecarboxylic acid, exhibiting immunomodulatory activities more potent than levamisole, and being nontoxic at the same time. In addition, an interesting correlation between the structure and immunological activity was found [14]. Subsequently, we synthesized a derivative characterized by an exceptionally high immunostimulatory activity [16]. That compound, RM11, was shown to strongly stimulate both humoral (antibody-forming cell number) and cellular (delayed type hypersensitivity) immune response in mice to sheep erythrocytes when given intraperitoneally prior to immunization. The compound was also effective when administered *per os*. The immunostimulatory properties of RM11 prompted us to investigate the ability of the compound to accelerate the function of the immune system of mice subjected to immune suppression by cyclophosphamide (CP). More specifically, we investigated the ability of RM11 to elevate the humoral and cellular immune response in CP-treated mice and to affect the blood cell composition in that model. In addition, the effects of RM11 on the secondary, humoral immune response, suppressed by methotrexate (MTX), and the phenotypes of T and B cells were studied.

Materials and Methods

Animals

CBA and BALB/c mice of both sexes, 8–12 weeks old, were used for the studies. The animals were fed a commercial, granulated food and water *ad libitum.* The Local Ethics Committee approved the studies.

Preparation of the compound for experiments

The compound (5 mg) was suspended in 0.3 ml of DMSO (Sigma), followed by a 20 min incubation in an ultrasonic bath. Then the compound was further diluted in 0.9% NaCl or culture medium. For the *in vivo* experiments, mice were treated with equivalent doses of DMSO.

The humoral immune response to sheep erythrocytes *in vivo*

Mice were given *ip* 0.2 ml of 2.5% SRBC suspension in 0.9% NaCl. Cyclophosphamide (CP), at the dose of 200 mg/kg, was given *ip* 35 days before immunization [4]. RM11 (10 μg/mouse) was administered *ip* to mice in 15 doses, on alternate days, before immunization. After 4 days the splenocytes were isolated and the number of antibody-forming cells (AFC) was determined by the local hemolysis assay [11]. The results are shown as the mean AFC values of 5 mice/group calculated per 10^6 viable splenocytes ± SE.

The secondary humoral immune response to SRBC *in vitro*

We followed a model described by us elsewhere [1]. Mice were primed with 0.2 ml of 1% SRBC suspension *ip*. After 4 days the splenocytes were isolated and a single cell suspension was prepared in a culture medium consisting of RPMI 1640, supplemented with 10% fetal calf serum, glutamine, sodium pyruvate, 2-mercaptoethanol and antibiotics. The cells were incubated in 24-well culture plates (5×10^6/ml/well) with addition of 50 μl of 0.005% SRBC. MTX was added to the cell cultures at a final concentration of 0.25–1 mM, 24 h after initiation/immunization of cultures. RM11 was added to the cell cultures at concentration of 10 μg/ml at the beginning of culture. After 4 days of culture, the number of AFC was determined. The results are shown as the mean values of AFC number from 4 wells ± SE, calculated per 10^6 viable cells.

The delayed type hypersensitivity to ovalbumin

Mice were sensitized with 10 μg of ovalbumin (OVA) emulsified in Freund's complete adjuvant into the tail base. After 4 days the mice were challenged with 50 μg of OVA in Freund's incomplete adjuvant into both hind foot pads. Following the next 24 h the delayed type hypersensitivity reaction was measured as the foot pad edema using a caliper with 0.05 mm accuracy. The background, nonspecific response was pro-

voked by administration of an eliciting dose of OVA to naive mice and was subtracted from the response of sensitized mice. CP was given to mice at the dose of 200 mg/kg, 14 days prior to sensitization of animals with OVA as described before [3]. One day following CP, mice were given 7 doses of RM11 (10 μg/mouse) on alternate days intraperitoneally. The results are shown as the mean values of 5 mice/ group (10 determinations) and expressed in DTH units (one unit = 0.1 mm) [9].

Preparation of blood smears

Blood smears were performed on microscopic slides and stained with Giemsa and May-Grunwald reagents. The preparations were analyzed by a histologist using 1,000 × magnification in immersion oil. Up to 100 cells were counted in two preparations for each mouse (five mice per group). The results are presented as the mean values (in percentage) for each cell type (lymphocytes, neutrophils, neutrophil precursors-band forms and eosinophils).

The phenotypic studies

Mice were given a single, *ip* 10 μg/ml dose of RM11. After 24 h the spleens were isolated and pressed through a nylon screen in a cold PBS. The cells were subsequently separated on a discontinuous Ficoll-uropoline gradient (density 1.071 g/ml), washed and resuspended in PBS with addition of 1% BSA. The following antibodies were used for cell staining: rat monoclonal anti-mouse CD4-FITC/CD8-RPE clone YTS 191.1/KT15 (lot 0605, SEROTEC) and rat monoclonal anti-mouse CD19-FITC/CD3-RPE, clone 6D5/KT3 (lot Dc 035, SEROTEC). The antibodies were used at dilutions suggested by the manufacturer. The stained cell suspensions were subjected to the phenotypic analysis using FACS (Becton Dickinson) according to CellQuest version 3.1.f program.

Statistical analysis

The results were presented as the mean values, mean ± standard error (SE) and mean ± standard deviation (SD). The Levene's test was used to determine the homogeneity of variance between groups. Analysis of variance (ANOVA) or Kruskal-Wallis test were applied to estimate the significance of the difference between groups. Significance was determined at $p \leq 0.05$. The statistical analysis was performed using STATISTICA for Windows statistical package.

Results

Partial reconstitution of the humoral immune response to sheep erythrocytes by RM11 in mice treated with cyclophosphamide

Mice were given a sublethal dose of CP and were treated with 15 doses of RM11 (10 μg *ip* on alternate days, the last dose 11 days before immunization with SRBC). Thirty five days following CP administration, the number of antibody-forming cells in the spleens was determined and shown in Figure 1. As expected [4], administration of CP resulted in a deep (84%) and long-term inhibition of the AFC number. Treatment of CP-immunocompromised mice with RM11 increased by 3.3-fold the immune response which constituted 61% of the response in untreated, control mice. Treatment of mice with RM11 alone was also significantly stimulatory.

Partial reconstitution of the cellular immune response (delayed type hypersensitivity) to ovalbumin by RM11 in mice treated with cyclophosphamide

Mice were given CP and treated with 7 doses of RM11 (10 μg *ip*, on alternate days, the last dose 1 day

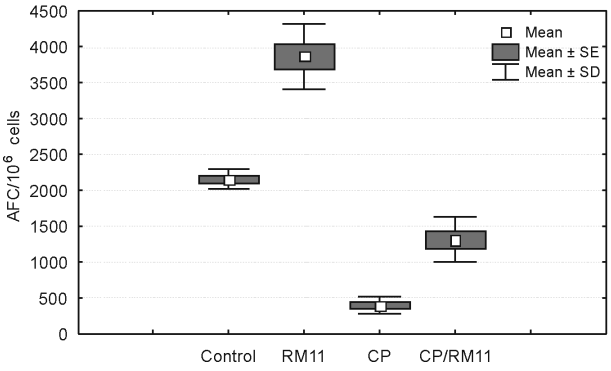

Fig. 1. Partial reconstitution of the humoral immune response *in vivo* to SRBC by RM11. Mice were given CP (200 mg/kg) and treated with 15 doses of RM11 (10 μg *ip* on alternate days). The AFC number was determined 35 days after CP administration. Statistics: Control *vs.* CP $p < 0.001$; Control *vs.* RM11 $p < 0.001$; CP *vs.* CP/RM11 $p < 0.001$

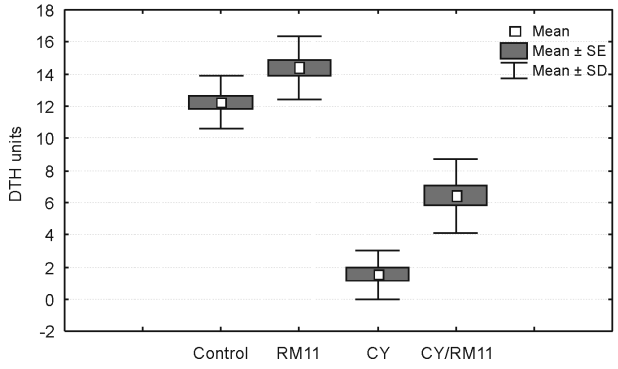

Fig. 2. Partial reconstitution of the cellular immune response *in vivo* to ovalbumin by RM11. Mice were given CP (200 mg/kg b.w.) and treated with 7 doses of RM11 (10 μg *ip* on alternate days). Mice were sensitized with OVA 14 days after CP administration and 4 days later the DTH reaction was elicited. Statistics: Control *vs.* CP $p < 0.001$; Control *vs.* RM11 $p < 0.02$; CP *vs.* CP/RM11 $p < 0.001$

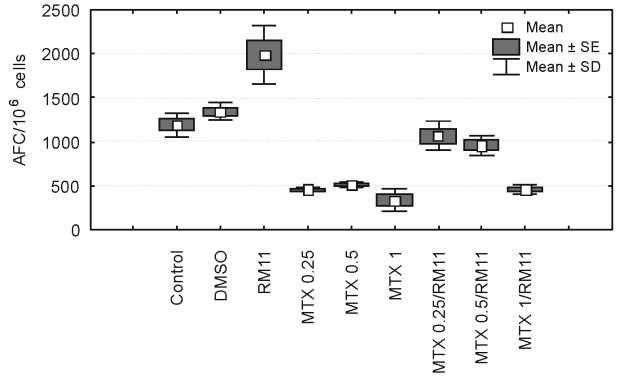

Fig. 3. RM11 counteracts suppressive actions of methotrexate in the secondary humoral immune response *in vitro* to SRBC. RM11 (10 μg/ml) was added to the cultures of splenocytes from SRBC-primed mice at the beginning of the 4-day incubation. MTX was added to the cultures at indicated doses, 24 h after initiation/immunization of the cell cultures. The AFC number was determined after 4 days. Statistics: Control *vs.* DMSO NS; DMSO *vs.* RM11 $p < 0.001$; Control *vs.* MTX 0.25 $p < 0.001$; Control *vs.* MTX 0.5 $p < 0.001$; Control *vs.* MTX 1 $p < 0.001$; MTX 0.25 *vs.* MTX 0.25/RM11 $p < 0.001$; MTX 0.5 *vs.* MTX 0.5/RM11 $p < 0.01$; MTX 1 *vs.* MTX 1/RM11 NS (NS – not significant)

before the eliciting dose of antigen). Mice were sensitized with OVA 14 days after CP administration and 4 days later the DTH reaction was elicited. The results (Fig. 2) revealed a strong impairment of the cellular response in CP-treated mice on day 14 (87.5% inhibition). Administration of RM11, however, significantly elevated the response (4.2-fold) which represented 52.3% of the value registered in control mice. Administration of RM11 alone was also stimulatory (a nonsignificant increase).

RM11 counteracts suppressive action of methotrexate on the secondary humoral immune response to SRBC *in vitro*

RM11 added (10 μg/ml) to the cultures of splenocytes isolated from SRBC-primed mice reversed the suppressive effect of MTX at concentration of 0.25 mM and significantly reconstituted the AFC numbers in cultures containing 0.5 mM or 1.0 mM concentrations of MTX (Fig. 3). The action of RM11 alone in that experimental model was also stimulatory.

The effect of RM11 on blood picture in cyclophosphamide-treated mice

Mice were given CP and treated with RM11 (10 μg/mouse on alternate days). The percentage of major blood cell types (neutrophil precursors, neutrophils, eosinophils and lymphocytes) was determined before (Fig. 4A) and 4 (Fig. 4B) and 11 days (Fig. 4C) after CP administration. The results demonstrated a typical neutropenia in CP-treated mice on day 4 (the nadir) (Fig. 4B). No effect of administration of two RM11 doses could be noted on that day. However, on day 11 after CP injection (Fig. 4C) the phenomenon of CP-mobilization of myelopoiesis was significant. It appeared that RM11 further enhanced the process of CP-induced myelopoiesis (the percentage of neutrophils was 37.4 *vs.* 51.5). More interestingly, the percentage of neutrophil precursors (bands) was even more elevated in mice treated with CP and RM11 (1.7 *vs.* 5.6).

The effect of RM11 on the phenotype of T and B cells

Table 1 shows the effects of a single *ip* administration of 10 μg of RM11 on the phenotype of T and B cells in the spleen after 24 h. The percentage of total ($CD3^+$) T cells as well as T cells bearing a phenotype of mature, effector T cells ($CD4^+$ and $CD8^+$) increased significantly compared with the appropriate solvent control (29.38 *vs.* 19.58, 18.98 *vs.* 15.01, and 8.89 *vs.* 4.58, respectively). On the other hand, the

percentage of cells expressing the pan B-cell marker fell from 71.76 to 62.12%.

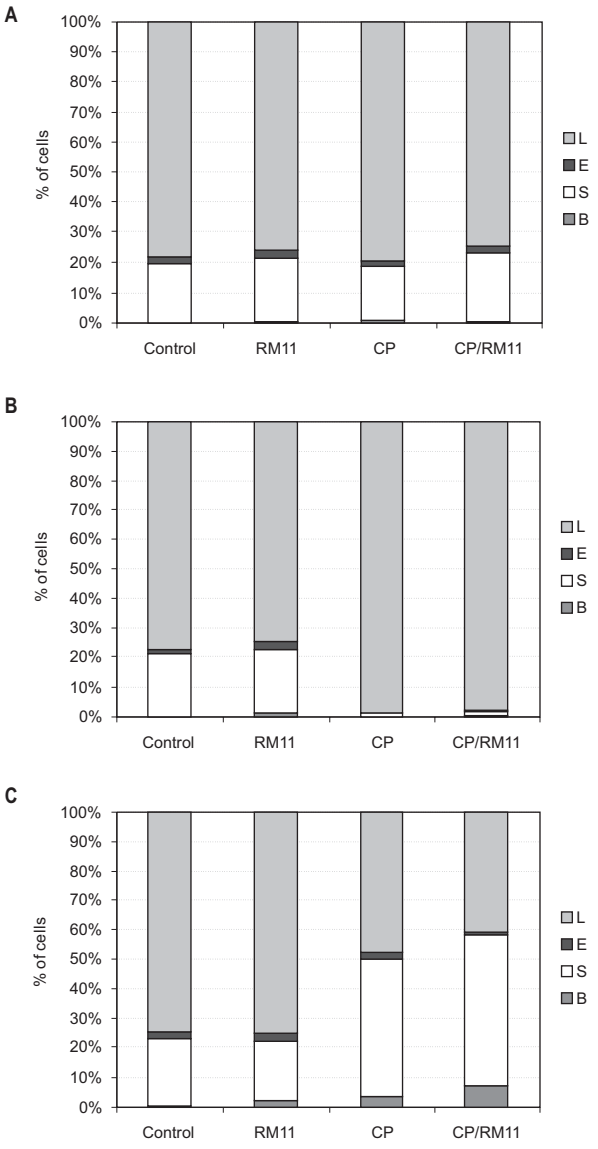

Fig. 4. Changes in the composition of the major blood cell types after treatment of mice with RM11 and cyclophosphamide. The determinations were performed on days: 0 **(A)**, 4 **(B)** and 11 **(C)** after CP administration. L – lymphocytes, E – eosinophils, S – mature neutrophils, B – bands (neutrophil precursors). Mice were given CP *ip* (200 mg/kg) followed by *ip* RM11 administration (10 μg/dose) on alternate days. Blood smears were analyzed just before CP injection, on day 4 and 11 after CP administration. Statistics: **Day 4** bands: no significant changes between groups; segments: Control *vs.* CP $p < 0.01$; Control *vs.* RM11 NS; CP *vs.* CP/RM11 NS; eosinophils: Control *vs.* CP NS; Control *vs.* RM11 NS; CP *vs.* CP/RM11 NS; lymphocytes: Control *vs.* CP $p < 0.01$; Control *vs.* RM11 NS; CP *vs.* CP/RM11 NS. **Day 11** bands: Control *vs.* CP NS; Control *vs.* RM11 NS; CP *vs.* CP/RM11 $p < 0.02$; segments: Control *vs.* CP $p < 0.001$; Control *vs.* RM11 NS; CP *vs.* CP/RM11 $p < 0.02$; eosinophils: no significant changes between groups; lymphocytes: Control *vs.* CP $p < 0.01$; Control *vs.* RM11 NS; CP *vs.* CP/RM11 $p < 0.01$ (NS – not significant)

Tab. 1. The effect of a single intraperitoneal administration of RM11 on the phenotype of T and B cells in the spleen

Phenotype	DMSO control	RM11
$CD3^+$	19.58 ± 2.27	29.38 ± 4.80*
$CD4^+$	15.01 ± 2.16	18.98 ± 0.96*
$CD8^+$	4.58 ± 1.48	8.89 ± 3.04*
$CD19^+$	71.76 ± 3.38	62.12 ± 5.07*

Mice were given 20 μg of RM11, *ip*. After 24 h the spleens were isolated and the phenotype of T and B lymphocytes was determined in a fluorocytometer. The content of respective cell phenotypes in the spleen is given in percentage (mean values from 5 mice) ± SE. All changes in comparison with DMSO control are statistically significant ($p < 0.05$)

Discussion

This study continues our preliminary evaluation of RM11 stimulatory action on the humoral and cellular immune response in mice [16]. In this study, we report the efficacy of RM11 in accelerating reconstitution of the cellular and humoral immune response in mice impaired by administration of cyclophosphamide and the secondary, humoral immune response *in vitro* suppressed by methotrexate. In addition, RM11 enhanced the process of myelopoiesis in CP-treated mice. In this investigation, we took advantage of the murine *in vivo* and *in vitro* models involving CP and methotrexate described by us recently [1–4] that optimized experimental conditions for testing of immunostimulatory actions of RM11.

The stimulatory effect of RM11 on both cellular and humoral immune response *in vivo* can be best explained by its action on the composition of T cell subpopulations in the spleen, although such a study was performed in mice not subjected to immunosuppression. We assumed that in normal mice even only one dose of the compound could elicit some phenotypic changes in T and B-cell populations. It appeared that a single *ip* injection of a small (20 μg/mouse) dose of RM11 led to a significant increase in the total T-cell population ($CD3^+$) and T cell subpopulation bearing T helper ($CD4^+$) cell phenotype in the spleen (Tab. 1). The action of RM11 was not restricted to recruitment of T helper cells but also to an increased generation of

T cells expressing CD8 marker (cytotoxic cells). The content of B cells expressing CD19 marker in the spleen fell significantly that may be associated with transition of $CD19^+$ B cells into more mature stage, i.e. IgD^+, $CD21^+$, $CD23^+$ since a portion of CD19-positive cells has features of immature B cells [21]. It is highly plausible that after depletion of T and B cells by cyclophosphamide [6, 13] RM11 may affect reconstitution of T and B-cell pool indirectly, by inducing relevant cytokines from cells of the reticuloendothelial system in the bone marrow and spleen. We showed, for example (unpublished data), that RM11 strongly up-regulated lipopolysaccharide-induced tumor necrosis factor-α (TNF-α) production by human blood mononuclear cells containing about 20% of monocytes. TNF-α is a cytokine clearly involved in the induction phase of the immune response and T-cell maturation [12, 20, 24], it seems, therefore, conceivable that this cytokine could promote the maturation of T cells in this study.

More interestingly, in the model of secondary immune response to SRBC *in vitro* RM11 at certain MTX concentrations was able to overcome the suppressive effect of MTX. Since the inhibitory action of MTX on the immune response is associated with induction of cell apoptosis [5] RM11 probably counteracts that action which may be, in addition, facilitated by the fact that memory T cells, responsible by generation of the secondary immune response, are less sensitive to apoptosis [22].

The stimulatory property of RM11 was not restricted only to the lymphocyte lineage since the compound enhanced mobilization of myelopoiesis (Fig. 4C), typically induced following initial neutropenia by CP, as demonstrated by us before [2]. That was evidenced not only by a significant increase in the mature neutrophil pool in CP-treated mice but also, to a greater extent, by appearance of immature neutrophil forms (5.57 *vs.* 1.7%). That effect shows that RM11 may act both on lympho- and myelopoiesis. In the latter case, however, RM11 is mainly a co-stimulator of myelopoiesis since the compound alone produced a much smaller stimulatory effect. Similar effect was also observed with another isoxazole, levamisole, in mice treated with 5-fluorouracil [8] although the authors claimed that those effects were not statistically significant. Levamisole, in addition, increased proliferation of bone marrow cells in mice given 5-fluorouracil [7].

Although in our initial report [16] we showed some enhancement of concanavalin-A-induced splenocyte proliferation by RM11, in this study we did not find any effect of RM11 on both concanavalin A and pokeweed mitogen-induced splenocyte proliferation. Likewise, RM11 did not stimulate mixed lymphocyte reaction (data not shown).

In conclusion, we propose that RM11, a potent immunostimulator, may be of potential value in patients undergoing chemotherapy to accelerate renewal of cells responsible for both innate (neutrophils) and acquired immunity (lymphocytes). In addition, the lack of stimulation of the mixed lymphocyte reaction is a desired property of RM11 since it should not generate graft versus host reaction after bone marrow transplant.

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