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EXPERIMENTAL STUDY

In vivo study of histamine H4 receptor in immunomodulation

Tripathi T[1], Shahid M[2], Khan HM[2], Siddiqui M[1], Khan RA[3], Shujatullah F[2], Moin S[1], Malik A[2]

Department of Biochemistry, Faculty of Medicine, Jawaharlal Nehru Medical College & Hospital, Aligarh Muslim University, Aligarh, India. **shahidsahar@yahoo.co.in**

Abstract: *Objective:* Recently accumulating evidence has highlighted the role of histamine in inflammation and immune reaction by histamine H4-receptor, however the role of histamine via H4-receptor in immunomodulation is still unclear. Therefore, the present study was designed to study the immunomodulatory role of histamine H4-receptor on antibody generation profile in rabbit.
Methods: The cohort study comprised of 108 rabbits in six groups. Each group consisted of 18 rabbits. Group I (negative control) remained non-immunized and received vehicle (sterile distilled water, 1 $mlkg^{-1}$ × b.i.d., s.c. for 10 days (3 days prior to immunization until 7 days after immunization)). Group II (positive control) received vehicle (1 $mlkg^{-1}$ × b.i.d., s.c. for 10 day), while group III–VI received histamine (100 μgkg^{-1} × b.i.d., s.c.), H4--agonist (clobenpropit dihydrobromide, 10 μgkg^{-1} × b.i.d., s.c.), and H4-antagonist (JNJ 7777120, 10 μgkg^{-1} × b.i.d., i.m.) and DMSO (control group for H4R-antagonist, 1 $mlkg^{-1}$ × b.i.d., i.m.) respectively for 10 days. Group II–VI were immunized with intravenous injection of sheep red blood cells (SRBC) on day 3. Immunological parameters [immunoglobulins (Ig), immunoglobulin M (IgM), and immunoglobulin G (IgG)] assessed by the whole SRBC-ELISA method and direct hemagglutination assay.
Results: Histamine could influence a detectable antibody response to SRBC as early as day 7 postimmunization (post-I), which lasted until day 58 post-I, whereas H4-receptor by H4R-antagonist treatment showed a similar profile of antibody (Ig, IgM, and IgG) generation as the positive control group. On the other hand, H4R-agonist treatment showed immunostimulant activity as compared to other experimental groups. The results were found statistically significant ($p<0.01$).
Conclusions: Histamine H4-receptor in biological system modulates immunological function and stimulates antibody production only by exogenously administered agonists not by endogenous histamine *(Tab. 1, Fig. 3, Ref. 26)*. Full Text in PDF *www.elis.sk.*
Key words: histamine receptors, immunomodulation, T-cell dependent antibody response, humoral immune response, H4-antagonist, H4-agonist, Rabbit, Sheep red blood cells.

Abbreviations: b.i.d. – two times per day, ELISA – enzyme linked immunosorbent assay, HA – hemagglutination assay, HR – histamine receptor, HRP – horseradish peroxidase, *im* – intramuscular, *sc* – subcutaneous, 2-ME – 2-mercaptoethanol, PBS – phosphate buffer saline, Post-I – post-immunization, Pre-I – pre-immunization, SRBC – sheep red blood cells, Th – T helper, TMB – tetramethyl benzidine, DMSO – Dimethyl sulphoxide.

[1]Department of Biochemistry, Faculty of Medicine, Jawaharlal Nehru Medical College & Hospital, Aligarh Muslim University, Aligarh, India, [2]Department of Microbiology, Faculty of Medicine, Jawaharlal Nehru Medical College & Hospital, Aligarh Muslim University, Aligarh, India and [3]Department of Pharmacology, Faculty of Medicine, Jawaharlal Nehru Medical College & Hospital, Aligarh Muslim University, Aligarh, India

Address for correspondence: M. Shahid, MD, PhD, MNZIMLS (New Zealand), Dept of Medical Microbiology, JN Medical College & Hospital, Aligarh Muslim University, Aligarh-202002, UP, India.
Phone: +91.571.2720382, Fax: +91.571.2721776

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Histamine regulates dendritic cells, T-lymphocytes, B-lymphocytes, as well as related antibody isotype responses (1). It's immunosuppressive and immunomodulatory effects on both humoral- and cell-mediated immune (HI and CMI, respectively) responses have been observed (2–5). Immunomodulation studies in rabbit model have showed that histamine has a short-term effect on antibody generation and the antibody (Immunoglobulins (Ig), immunoglobulin-M (IgM), and IgG) production in vivo were affected by the concentration of histamine (4,5). Histamine receptors (H1R and H2R) have been shown to enhance delayed hypersensitivity and antibody mediated immune responses in many pathological processes regulating several essential events in allergies and autoimmune diseases in experimental animals, especially in knock out mice (either H1R- or H2R-deficient) (6–8). Histamine and HRs (H1R and H2R)-agonist enhances antibody production by triggering the histamine receptors (H1R and H2R), whereas both H1R-antagonist and H2R-antagonist positively or negatively regulate the antibody profile. Anti-IgM is increased in H2R-antagonist treated rabbits and it is diminished in H1R-antagonist treated rabbits. H1R-antagonist treated rabbits displays diminished antibody production against a T cell-dependent antigen-SRBC as compared to H2R-antagonist treated and control rabbits (9). Jutel et al (10) studied that tripelennamine (a H1R-antagonist) inhib-

ited histamine binding in Th1 but not in Th2 cells and showed predominant H1R expression on Th1 cells. Neither ranitidine (a H2R-antagonist) nor clobenpropit (a H3R-antagonist or a H4R-partial agonist) had any impact on histamine binding to Th1 cells. Their study demonstrated the expression of H1R on Th1 cells and H2R on Th2 cells by antibodies generated against the H1R and H2R (10). Clobenpropit has two different pA2 values i.e. pA2 = 7.9 (EC50 72 nM) for H4R-agonist and pA2 = 9.9 for H3R antagonist (11). However, clobenpropit has been used as H4R-agonist that mimics the histamine effect in inducing change of shape of eosinophils (12). Moreover, histopathological and biochemical study of clobenpropit demonstrated its agonist property in rabbit and showed that it causes binucleated hepatocytes and Kupffer cells prominence (13).

Recently accumulating evidence has highlighted the histamine role in inflammation and immune reaction by histamine H4-receptor (1), however the histamine role via H4-receptor in immunomodulation is still unclear. Moreover, the studies in rabbit model are elementary, and the existing studies have demonstrated immunomodulatory role studying only single blood samples taken after immunization of the animals [except our earlier reports on immunomodulatory profile (4, 5, 9, 11, 14–16).

Keeping in view the above facts, especially the paucity of literature (i.e., immunomodulatory role of histamine H4 receptor, defining the co-relation of histamine H4 receptor-agonist/-antagonist in immune regulation, and fragmentary histamine literature describing existing immunomodulatory role of histamine *in vivo* system, the present hypothesis was designed.

Materials and methods

Experimental design

To evaluate the systemic antibody response, 108 (54 Male and 54 Female) New Zealand adult healthy rabbits of either sex weighing 1 – 1.5 kg were divided into six treatment groups. Each group contained 18 rabbits (1:1 male to female ratio). Group I (negative control) remained non-immunized and received only vehicle (sterile distilled water, 1 $mlkg^{-1}$ × b.i.d.). Group II was vehicle (sterile distilled water, 1 $mlkg^{-1}$ × b.i.d.) treated and immunized as a positive control. Group III was histamine treated and immunized, Group IV was DMSO treated (control group for H4R-antagonist) and immunized, group V was H4R-agonist treated and immunized, group VI was H4R-antagonist treated and immunized. All animals were housed in well-maintained animal facility at the central animal house, J. N. Medical College & Hospital, Aligarh Muslim University, Aligarh, in the Bioresources unit under a 12 hr light/dark cycle, temperature (22±2 °C), and were allowed free access to standard laboratory diet including green vegetables and tap water until experimentation. Each animal was used only once. All studies were carried out during the light cycle and were approved by the Institutional Animal Ethical Committee.

Materials

All materials were obtained from the following manufacturers: Monoclonal-anti-rabbit-immunoglobulins-horseradish peroxidase (HRP) conjugate and monoclonal-anti-rabbit-IgG-HRP conjugate from Sigma (USA), anti-rabbit-IgM-HRP conjugate from G Biosciences from Maryland heights (USA), tetramethyl benzidine (TMB) and TMB diluent from J. Mitra and Co. (India), Polystyrene MaxiSorp microtitre flat and round bottom ELISA plates from NUNC (Denmark), Glutaraldehyde solution from Central Drug House (India), Skim milk from Nestle India Ltd. (New Delhi), 2-mercaptoethanol (2-ME) from Merck KGaA, Darmstadt (Germany) and DMSO obtained from Qualigen**,** Glaxo**,** India. All chemicals were of analytical grade.

Drugs

In the present study, the following drugs were used: histamine dihydrochloride by Himedia laboratories Pvt Limited, India; H4R-agonist (clobenpropit dihydrobromide) kindly donated by Tocris Bioscience, Tocris Cookson Ltd., United Kingdom; and H4R-antagonist (JNJ 7777120) purchased from Sigma (USA).

Dosage regimen

Histamine (100 µg/kg), clobenpropit dihydrobromide (10 µg/kg) were administered twice in a day through subcutaneous (s.c.) route, and JNJ 7777120 (10 µg/kg) and DMSO (1 ml/kg) were administered twice in a day [(2 hourly (8:00 am to 8:00 pm)) through intramuscular (i.m.) route; starting from three days prior to immunization until 7 days after immunization. All doses referred to the weight of the salts used.

Antigen

Sheep blood diluted 1:1 in sterile Alsevier's solution was obtained from Department of Microbiology, J. N. Medical College & Hospital, A.M.U., Aligarh, and washed with PBS (10 mM sodium phosphate buffer containing 150 mM NaCl, pH-7.4) thrice by centrifugation. The cell suspensions were adjusted to the desired concentration in terms of hemoglobin, lysis of a 1 % SRBC suspension (2×10^8 cells/ml) with 14 volumes of 0.1 % Na_2CO_3 develops an optical density of 0.135 at 541 nm in a spectrophotometer (Systronics, UV visible double beam spectrophotometer-2101, India), as described Franzl (17). Finally the concentration was adjusted to 5 % (1×10^9 cells/ml) in PBS for immunization before the use.

Immunization of rabbits

The rabbits in all experimental groups (II-VI) were immunized intravenously via marginal ear vein with 1 ml of 5 % (1×10^9 cells/ml) sheep red blood cells (SRBC) in PBS.

Sample collection

To determine the systemic antibody response, blood samples were collected from rabbits through the marginal ear veins into labeled sterile bottles prior to immunization (day 0), as well as on days 7-, 14-, 21-, 28-, and 58- post-immunization. Blood samples were kept at room temperature for 2 hr and then at 4 ºC overnight. Blood samples were centrifuged for 10 minutes at 580×g, and serum was isolated and heated at 56 ºC for 30 minutes to inactivate complement proteins and stored in aliquots containing sodium azide as preservative at –20 ºC (4,5,8,10,14,15) till tested further.

Serological analysis

Hemagglutination assay (HA)

To determine the antibodies response to SRBC, a direct hemagglutination technique was used (9, 14, 15, 18). Briefly, 100 µL of PBS was dispensed into each well of a round bottomed 96-well microplate. Serum sample (100 µL) were then added using serial two-fold dilutions (1:2, 1:4, 1:8, 1:16, 1:32) in the wells from columns 2 to 12. The first column (PBS only) of wells was considered blank. Then, 100 µL of 2 % sheep red blood cells (SRBC) in PBS was added to all wells to make a final volume of 200 µL. Subsequently, the plates were shaken for 1 minutes and incubated at 37 °C for 1 hr, and then overnight at 4 °C to determine agglutination titers. A positive result was recorded when at least 50% SRBC agglutination was observed. To measure anti-SRBC-immunoglobulin-M (IgM) and immunoglobulin-G (IgG), serum sample were treated with 0.2M 2-mercaptoethanol (2-ME) for 1 hr at 37 °C. This treatment inactivates IgM antibody, and as a result, hemagglutination observed after treatment with 2-ME is mostly due to the presence of IgG antibody. The difference between total antibodies (Ig) and IgG antibody titers were taken as the titers of IgM antibody.

Enzyme linked immunosorbent assay (ELISA) using whole SRBC

To determine the SRBC-specific-immunoglobulins (Ig), SRBC-specific-IgM and SRBC-specific-IgG response, the whole SRBC-enzyme linked immunosorbent assay (ELISA) was carried out on polystyrene plates (4, 5, 9, 11, 14–16). Briefly, polystyrene MaxiSorp immunoplates were coated with SRBC suspension (5×10^6/100 µL PBS [10 mM sodium phosphate buffer containing 150 mM NaCl, pH-7.4]). The plates were held overnight at 4ºC. Each sample was coated in duplicate and half of the plates served as control devoid of antigen coating. Without disturbing the cell layer, 20 µL of 1.8 % glutaraldehyde solution was then gently added to plates inoculated with SRBC and the plates were held at 25 °C for 30 min. Unbound SRBC was washed four times with 200 µL of PBS and non-specific binding sites were blocked with 1 % fat-free milk in PBS for 2 hr at 37 °C. After incubation, the plates were washed four times with 200 µL of PBS. Each rabbit serum diluted 1:100 in PBS (100 µLwell^{-1}) was adsorbed for 1.5 hr at 37 °C, and then overnight at 4ºC followed by washing as earlier. The secondary antibody, HRP conjugated monoclonal-anti-rabbit-immunoglobulins, monoclonal-anti-rabbit-IgM and monoclonal-anti-rabbit-IgG was then added (100 µLwell^{-1}) in respective plates and incubated at 37 °C for 1 hr. The washing stage was repeated as before and 100 µLwell^{-1} TMB substrate was added and the plates were incubated at 25 °C for 1 hr. The enzymatic reaction was stopped by adding 50 µLwell^{-1} of 5 % H_2SO_4. The absorbance (A) was determined at 405 nm on an automatic ELISA plate reader (Micro scan MS5608A, ECIL, India). Each rabbit serum sample was run in duplicate. The control wells were treated similarly but were devoid of antigen. Results were expressed as a mean of $A_{\text{test- control}}$.

Statistical analysis

Data were summarized as the mean ± SD. Groups were compared by using repeated measures (subjects within groups) two way analysis of variance (ANOVA) followed by the Newman-Keuls post hoc test. A two-tailed ($\alpha = 2$) probability $p<0.05$ was considered to be statistically significant. Analyses were performed on SPSS for Windows (version 12.0, Inc., Chicago, IL).

Results

To evaluate the effects of histamine H4 receptor-agonist and antagonist on the immunomodulation, antibody-mediated responses to SRBC were assessed. Total serum immunoglobulins (Ig), total immunoglobulin-M (IgM) and total immunoglobulin G (IgG) generation profiles were studied *in vivo* in six experimental groups on day 0 (pre-immunization (pre-I)) and days 7, 14, 21, 28, and 58 (post-immunization (post-I)).

Profile of total anti-SRBC-immunoglobulins (Ig) production

The profile of total anti-SRBC-Ig titer was studied by the whole SRBC-ELISA method (4, 5, 9, 11, 14-16) (Fig. 1) and direct hemagglutination assay (9, 14, 15, 18) (Tab. 1). The observed profiles were similar by ELISA and HA assay, however the results were found comparatively more significant by ELISA as opposed to HA. No antibody response was detected in all groups (control and drug treated) on day 0 (pre-I). There was an initial increase and subsequent decrease in total serum antibody titer over a time span of 58 days in all the groups, and was found statistically significant on each post-I days. Two-way ANOVA (analysis of variance) has revealed that the effect of days, effect of treatment (drugs) and effect of interaction (drugs and days) were statistically significant $p<0.01$, respectively. By the day 7- post-I, antibody titer was significantly high, however it obtained a peak on day 14- post-I and by days 21-, 28-, and 58- post-I, there was a gradual decrease or a plateau in positive control and DMSO treated as compared to H4R-agonist treated, H4R-antagonist treated, and histamine treated groups (Fig. 1 and Tab. 1). More extensive evaluation revealed that anti-SRBC-immunoglobulins (Ig) raised steeply up to 7 days post-I and there was a significant decrease in H4R-agonist treated and histamine treated group as compared to other groups. While in H4R-antagonist treated group, anti-SRBC-Ig increased gradually on days 7- and 14- post-I, and obtained a high peak on day 21- post-I. Moreover, on days 28- and 58- post-I, there was a gradual decrease as compared to other groups.

Histamine showed an initial enhancement and later a suppression of anti-SRBC-Ig production profile during the whole study as compared to H4R-antagonist, positive control group and DMSO treated groups only (significant increase of total serum anti-SRBC-Ig level was noticed on day 7 post-I ($p<0.01$), and suppression of serum anti-SRBC-Ig levels on days 14 and 21 post-I ($p<0.01$ each for H4R-antagonist, positive control and DMSO treated groups). On days 28 and 58 post-I, the results were found statistically significant when compared to H4R-antagonist ($p<0.01$); conversely the results were same in comparison to positive control and DMSO treated groups. DMSO-treated group served as the control group for H4R-antagonist (JNJ 7777120) and this group showed a similar pattern of anti-SRBC-Ig generation profile to positive control.

Tab. 1. Immunomodulatory study of histamine and H4R-agonist/-antagonist treated rabbits determined by hemagglutination assay.

Groups of Experimental Rabbits (n = 18)	Mean antibody titre ± s.d.*																	
	Total Anti-SRBC-Immunoglobulins♦						Anti-SRBC-Immunoglobulin-M♦♦						Anti-SRBC-Immunoglobulin-G♦♦♦					
	Pre-I	Post-I					Pre-I	Post-I					Pre-I	Post-I				
	0 Day	7th Day	14th Day	21st Day	28th Day	58th Day	0 Day	7th Day	14th Day	21st Day	28th Day	58th Day	0 Day	7th Day	14th Day	21st Day	28th Day	58th Day
Negative Control (Group I)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Positive Control (Group II)	0.00 ± 0.00	4.67 ± 0.52	5.00 ± 0.63	4.00 ± 0.89	3.50 ± 0.55	2.33 ± 0.52	0.00 ± 0.00	4.13 ± 0.88	4.00 ± 0.63	2.33 ± 1.21	1.50 ± 0.52	0.00 ± 0.00	0.00 ± 0.00	2.33 ± 0.51	4.00 ± 0.63	3.33 ± 1.03	3.00 ± 0.89	2.33 ± 0.52
Histamine (Group III)	0.00 ± 0.00	5.87 ± 0.63	4.17 ± 0.41	3.83 ± 0.41	3.47 ± 0.52	1.67 ± 0.51	0.00 ± 0.00	5.29 ± 0.14	3.17 ± 0.41	2.33 ± 1.19	1.67 ± 0.63	0.00 ± 0.00	0.00 ± 0.00	4.27 ± 0.17	3.17 ± 0.41	3.00 ± 0.63	2.17 ± 0.87	1.67 ± 0.51
DMSO (Group IV)	0.00 ± 0.00	4.17 ± 0.33	4.87 ± 0.67	3.93 ± 0.82	3.33 ± 0.53	2.17 ± 0.52	0.00 ± 0.00	4.00 ± 0.78	4.00 ± 0.53	2.17 ± 1.17	1.33 ± 0.43	0.00 ± 0.00	0.00 ± 0.00	2.17 ± 0.53	3.87 ± 0.67	3.33 ± 1.00	2.67 ± 0.68	2.17 ± 0.47
H4R-agonist (Group V) (clobenpropit)	0.00 ± 0.00	5.17 ± 0.41	4.83 ± 0.41	4.33 ± 0.51	4.17 ± 0.40	3.00 ± 0.63	0.00 ± 0.00	4.97 ± 0.40	4.41 ± 0.41	3.43 ± 0.69	1.00 ± 0.54	0.00 ± 0.00	0.00 ± 0.00	2.17 ± 0.41	2.83 ± 0.41	3.17 ± 0.41	3.83 ± 0.75	3.00 ± 0.63
H4R-antagonist (Group VI) (JNJ 7777120)	0.00 ± 0.00	4.33 ± 0.52	4.84 ± 0.52	5.67 ± 0.52	4.67 ± 1.03	3.50 ± 0.55	0.00 ± 0.00	3.99 ± 0.63	4.30 ± 0.52	3.90 ± 0.59	2.10 ± 0.82	0.00 ± 0.00	0.00 ± 0.00	2.17 ± 0.40	3.87 ± 0.52	4.16 ± 0.98	4.33 ± 0.82	3.33 ± 0.52

*Results demonstrate mean ± s.d. of –log2 titers of three independent experiments each with six rabbits. Two-way ANOVA followed by Newman-Keuls post hoc test revealed that ♦the effect of treatments ($F=182.150$, $DF=5,102$; $p<0.01$), days ($F=3996.643$, $DF=5,510$; $p<0.01$) and the interaction (treatments × days) effect ($F=222.283$, $DF=25,510$; $p<0.01$) on SRBC were found to be significant; ♦♦the effect of treatments ($F=92.042$, $DF=5,102$; $p<0.01$), days ($F=1797.997$, $DF=5,510$; $p<0.01$) and the interaction (treatments × days) effect ($F=96.129$, $DF=25,510$; $p<0.01$) on SRBC were found to be significant; ♦♦♦the effect of treatments ($F=86.209$, $DF=5,102$; $p<0.01$), days ($F=1414.997$, $DF=5,510$; $p<0.01$) and the interaction (treatments × days) effect ($F=124.194$, $DF=25,510$; $p<0.01$) on SRBC were found to be significant.

H4R-agonist-treated group showed a significant ($p<0.01$) enhanced anti-SRBC-Ig profile as opposed to the positive control, DMSO treated, H4R-antagonist treated, and histamine treated groups. However, H4R-antagonist studies showed a similar anti-SRBC-Ig level to positive control and DMSO treated on day 7 post-I but showed a significant ($p<0.01$) suppression as compared to the histamine treated group. While on day 14 post-I it showed an insignificant suppressed Ig level as compared to the positive control group, but a significant ($p<0.01$) enhancement as compared to the histamine treated group. Furthermore, the H4R-antagonist treated group showed a significant ($p<0.01$) enhancement of antibody generation level on day 21, 28, and 58 post-I as compared to the positive control, DMSO treated, and histamine-treated groups. No antibody response was noticed in the group I (negative control) during the whole study period (Fig. 1 and Tab. 1).

Profile of total anti-SRBC-immunoglobulin-M (IgM) production

Anti-SRBC IgM was determined by the whole SRBC-ELISA method (4, 5, 9, 11, 14–16) (Fig. 2) and direct hemagglutination assay (9, 14, 15, 18) (Tab. 1). No IgM response was observed in all groups on day 0 pre-I, however there was an initial increase and then a gradual decrease in serum IgM titer over time in the positive control, DMSO treated, and drug treated groups. Two-way ANOVA measures revealed a significant effect of days ($p<0.01$), significant effect of treatment (drugs) ($p<0.01$) and significant effect of interaction (drugs and days) ($p<0.01$). By day 7 post-I, the IgM titer increased and obtained the highest peak, but by days 14, 21, 28, and 58 post-I there was a gradual decrease in positive control, DMSO-treated, histamine treated, and H4R-agonist treated groups.

While, by the day 7 post-I, the antibody titer was gradually increased and obtained a peak on day 14 post-I, and by days 21, 28, and 58 post-I there was a gradual decrease in the H4R-antagonist treated group (Fig. 2 and Tab. 1). The DMSO treated group showed a similar pattern of anti-SRBC-IgM generation profile to positive control group over a time span of 58 days.

In the histamine treated group, anti-SRBC-IgM raised steeply up to 7 day post-I, significantly ($p<0.01$) enhanced as compared to the positive control, DMSO treated, and H4R antagonist), and there was a significant ($p<0.01$) decrease on days 14 and 21 post-I as compared to the positive control, DMSO treated, and H4R-antagonist treated group while on day 28 post-I, it was significantly ($p<0.01$) suppressed to H4R antagonist, however on day 58 post-I, it showed a similar anti-SRBC-IgM level to the positive control, DMSO treated, and H4R antagonist groups.

The H4R-agonist treated group showed a significant ($p<0.01$) enhancement of anti-SRBC-IgM generation profile as compared to the positive control, DMSO treated, H4R-antagonist treated, and histamine-treated groups. While the H4R-antagonist treated group showed an insignificant suppression as opposed to the positive control and DMSO treated, it showed a significant ($p< .01$) suppression as compared to the histamine treated group. Furthermore, the H4R-antagonist treated group demonstrated a significantly ($p<0.01$) enhanced anti-SRBC-IgM levels on days 14, 21, and 28 post-I, however with an insignificant enhancement on day 58 post-I, as compared to the positive control, DMSO treated and histamine treated groups. No antibody response was noticed in the Group I (negative control) during the whole study period (Fig. 2 and Tab. 1).

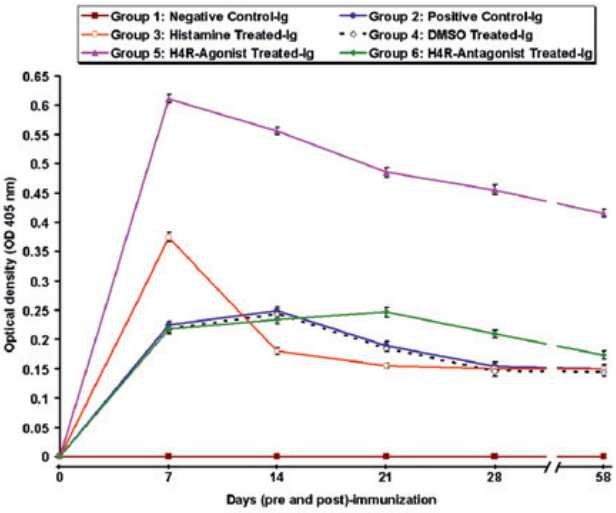

Fig. 1. SRBC-specific Immunoglobulins (Ig) production titers in H4R-agonist/antagonist-treated rabbits by whole SRBC-ELISA method in duplicate 1:100 diluted sera. The results demonstrate mean ± SD of three experiments each with six rabbits. Two-way ANOVA followed by Newman-Keuls post hoc test revealed that the effect of treatments (F = 13309.637, DF = 5,102; p<0.01) and days (F = 89815.206, DF = 5,510; p<0.01) on SRBC were statistically significant. The interaction (treatments × days) effect of (F = 9480.445, DF = 25,510; p<0.01) these on SRBC were also found to be significant.

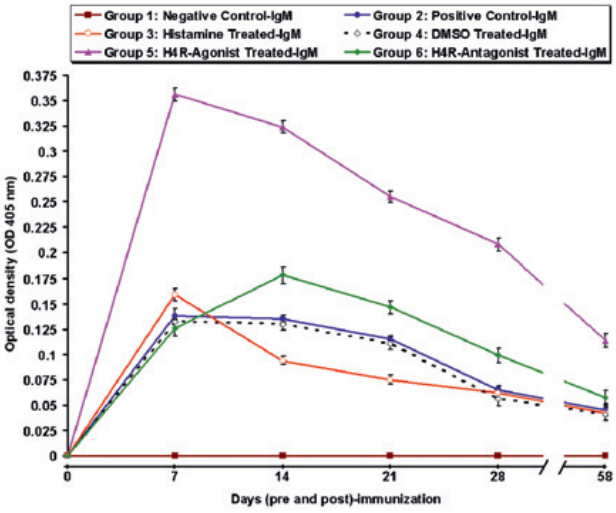

Fig. 2. SRBC-specific Immunoglobulin-M (IgM) production titers in H4R-agonist/antagonist-treated rabbits by whole SRBC-ELISA method in duplicate 1:100 diluted sera. The results demonstrate mean ± SD of three experiments each with six rabbits. Two-way ANOVA followed by Newman-Keuls post hoc test revealed that the effect of treatments (F = 5844.533, DF = 5,102; p<0.01) and days (F = 21533.286, DF = 5,510; p<0.01) on SRBC were statistically significant. The interaction (treatments × days) effect of (F = 2151.403, DF = 25,510; p<0.01) these on SRBC were also found to be significant.

Profile of total anti-SRBC-immunoglobulin-G (IgG) production

The profile of total anti-SRBC-IgG titer was studied by the whole SRBC-ELISA method (4, 5, 9, 11, 14–16) (Fig. 3) and direct hemagglutination assay (9, 14, 15, 18) (Tab. 1). The observed profiles were similar by ELISA and HA assays. No antibody response was detected in all groups at day 0 (pre-I). More extensive evaluation revealed that anti-SRBC-IgG in the histamine treated group and H4R-agonist treated group raised steeply up to 7 days post-I. But by days 14, 21, 28, and 58 post-I, there was a decrease or a plateau in anti-SRBC-IgG generation levels. While in the groups (positive control and DMSO treated), anti-SRBC-IgG generation titer was gradually enhance and obtained a high peak on day 14 post-I and by days 21, 28, and 58 post-I, there was a plateau. However, in the H4R-antagonist treated group, the anti-SRBC-IgG titer gradually increased up to 28 post-I and then decreased or maintained a plateau on day 58-post-I.

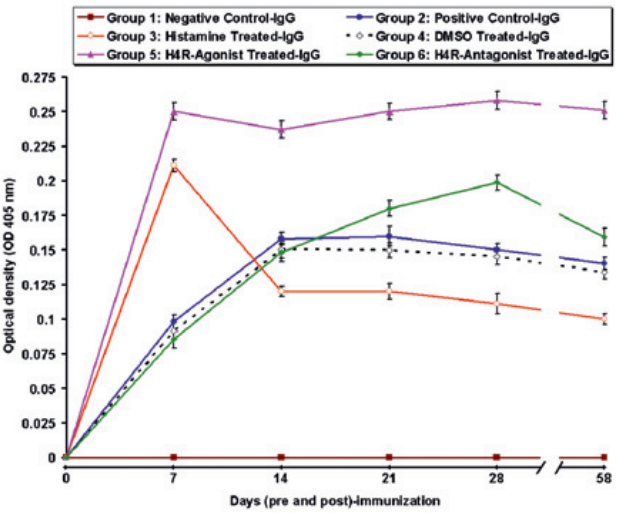

Fig. 3. SRBC-specific Immunoglobulin-G (IgG) production titers in H4R-agonist/antagonist-treated rabbits by whole SRBC-ELISA method in duplicate 1:100 diluted sera. The results demonstrate mean ± SD of three experiments each with six rabbits. Two-way ANOVA followed by Newman-Keuls post hoc test revealed that the effect of treatments (F = 5463.127, DF = 5,102; p<0.01) and days (F = 5888.457, DF = 5,510; p<0.01) on SRBC were statistically significant. The interaction (treatments × days) effect of (F = 581.692, DF = 25,510; p<0.01) these on SRBC were also found to be significant.

The H4R-agonist treated group showed an enhancement of anti-SRBC-IgG generation profile as compared to all groups over a time span of 58 days. The H4R-antagonist study demonstrated an insignificant suppression (as compared to the positive control and DMSO treated groups on day 7 and 14 post-I) and a significant (p<0.01) suppression (as compared to the histamine treated on day 7 post-I) of anti-SRBC-IgG level. However, this group further showed a significant (p< .01) enhancement of anti-SRBC-IgG level on days 21, 28, and 58 post-I as compared to DMSO treated and positive control groups, while it showed a significant (p<0.01) enhancement of anti-SRBC-IgG level on day 14, 21, 28, and 58 post-I as compared to histamine treated group only.

Two-way ANOVA revealed a significant effect of days (p<0.01), significant effect of treatment (drugs) (p<0.01) and significant effect of interaction (drugs and days) (p<0.01). No anti-SRBC-IgG response was noticed in the Group I (negative control) over a time span of 58 days (Fig. 3 and Tab. 1).

Discussion

In the present study, we studied total serum antibody (Ig), IgM, and IgG generation profile against SRBC, and T lymphocyte-dependent test antigen (19, 20), modulated by endogenous histamine in the positive control (untreated), H4R-agonist (clobenpropit dihydrobromide) treated, H4R-antagonist (JNJ 7777120) treated, DMSO treated, and histamine treated groups.

According to the document of the International Conference on Harmonization (ICH) S8 Guideline on Immunotoxicity Testing for Pharmaceuticals (adapted by EU in 2005, and by the FDA and MHLW in 2006), the evaluation of a primary antibody response to a T-lymphocyte-dependent antigen (e.g., SRBC or Keyhole limpet hemocyanin (KLH)) is recommended as one of the most sensitive immune tests following chemical exposures (19). The T-lymphocyte-dependent antibody response assay is a sensitive indicator of immunological integrity. When SRBC is used as the particular T-lymphocyte-dependent antigen, this response requires the coordinated interaction of various immune system cells (i.e., antigen-presenting cells, T-lymphocytes, and B-lymphocytes) (19, 21). Several studies have demonstrated the modulation of antibody-mediated immune response against SRBCs following chemical exposures (i.e., immunomodulatory and immunosuppressive agents) (22, 23).

Histamine, on releasing, endogenously modulates different physiological and pathological reactions via its all four receptors (H1R, H2R, H3R, and H4R) (1, 10, 24). In immunological reaction, histamine and HRs (H1R and H2R) agonist/antagonist affects B-cell antibody production as a co-stimulatory receptor on B-cells (2, 9, 25–27). To provide an exact evidence relating our investigations to *in vivo* immunoregulatory processes, we used healthy rabbits with and without histamine, H4R-agonist/-antagonist and DMSO treatments. Here we demonstrate that H4R-agonist/-antagonist treated rabbits were characterized by a marked modulation of the immune response as compared to the positive control (untreated), DMSO, and histamine treated rabbits.

This study demonstrates that the histamine released from immunological stimuli from effector cells *in vivo* (1), could influence a detectable antibody response to SRBC as noticed in our previous studies (4, 5, 9, 14). Moreover, the present study on *in vivo* immunomodulatory processes showed an enhanced generation profile of total anti-SRBC-Ig, anti-SRBC-IgM, and anti-SRBC-IgG in H4R-agonist treated group. In contrast, H4R-antagonist treated group demonstrated initially a suppressed and then later an enhanced profile of total anti-SRBC-Ig, IgM, and IgG over the time span of 58 days.

These results demonstrated that H4-receptor on inhibition by H4R-antagonist showed no modulatory activity of antibody (immunoglobulins (Ig), IgM and IgG) generation similar to positive control. While H4-receptor on stimulation by H4R agonist showed an immunostimulant activity (enhanced the antibody generation levels as compared to the H4R-antagonist and positive control).

Conclusion

Histamine H4-receptor in biological system modulates immunological function and stimulates antibody production only by exogenously administered agonists not by endogenous histamine.

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