

A complex of histamine/mouse γ -globulin preferentially inhibits allergen-induced peritoneal accumulation of eosinophils, but not neutrophils, in mice

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A complex of histamine/human γ -globulin (HhG) has been widely used in Japan for more than 25 years as a nonspecific hyposensitization drug in the treatment of allergic diseases. It has been reported that HhG decreases the number of eosinophils in the nasal secretions and peripheral blood of patients with allergy. In this study we used a mouse system to explore the possibility that HhG may actively inhibit the accumulation of eosinophils at inflammation sites. A complex of 0.15 μ g of histamine dihydrochloride/12 mg of mouse γ -globulin (HmG) was incubated for 2 hours in saline solution in the normal fashion for HhG. HmG at 50 to 150 mg/kg/day inhibited the peritoneal accumulation of eosinophils induced by ragweed pollen in BALB/c mice in a dose-dependent fashion when the HmG was administered subcutaneously six times during a 20-day sensitization period. The inhibitory effect of HmG on this eosinophil accumulation was significant at 24 and 48 hours after challenge, but HmG had no effect on neutrophil accumulation. Complexes of serotonin/mouse γ -globulin (m γ G), glutamine/m γ G, and histamine dihydrochloride (His)/mouse albumin had no inhibitory effect when administered in the same way. The optimum combination ratio was between 0.15 μ g of His/12 mg of m γ G and 0.015 μ g of His/12 mg of m γ G for this eosinophil inhibition. Moreover, a 1- to 2-hour incubation period of His and m γ G was needed to induce a plateau inhibition of the eosinophil accumulation. These results in mice suggest that HhG may actively inhibit allergen-induced eosinophil accumulation, which may be therapeutically useful in the treatment of allergic disease. (*J Allergy Clin Immunol* 1997;100:809-16.)

Key words: Histamine, γ -globulin, eosinophil accumulation, ragweed pollen, complex of histamine/human γ -globulin, complex of histamine/mouse γ -globulin

Abbreviations used

His:	Histamine dihydrochloride
m γ G:	Mouse γ -globulin
HhG:	Complex of histamine/human γ -globulin
HmG:	Complex of histamine/mouse γ -globulin

Accumulation of eosinophils at inflammation sites is one of the characteristic features of allergic inflammation. It has been observed that eosinophils are accumulated and activated at inflammation sites by a variety of factors, including leukotriene B₄,^{1,2} platelet activating factor,³⁻⁶ IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor.⁷⁻¹⁵ The activated eosinophils have been shown to release cytotoxic substances such as major basic protein, eosinophil cationic protein, and eosinophil peroxidase, with consequent damage to the mucosal epithelium in allergic patients with, for example, atopic asthma, allergic rhinitis, or atopic dermatitis.¹⁶⁻²⁵ These findings suggest that the accumulation and/or activation of eosinophils may play a crucial role in the development and maintenance of allergic inflammation. For this reason, drugs capable of inhibiting the maturation, accumulation, and/or activation of eosinophils can be expected to be useful for the treatment of allergic disease.

A complex of histamine/human γ -globulin (HhG), which has been widely used for more than 25 years in Japan as a nonspecific hyposensitization drug with a low incidence of side effects, is useful for the treatment of allergic diseases such as allergic rhinitis, atopic dermatitis, and bronchial asthma. In two different kinds of double-blind studies performed with a total of 264 patients with allergic rhinitis, HhG injected subcutaneously twice a week for 6 weeks was found to significantly improve the score of clinical symptoms such as sneezing, nasal blockage, and color of the mucus membrane (when compared with the effect of the active placebo, human γ -globulin).^{26,27} Side effects were not observed in any patient in these studies. Another double-blind study performed with 129 patients with bronchial asthma indicated that subcutaneous injection of HhG twice a

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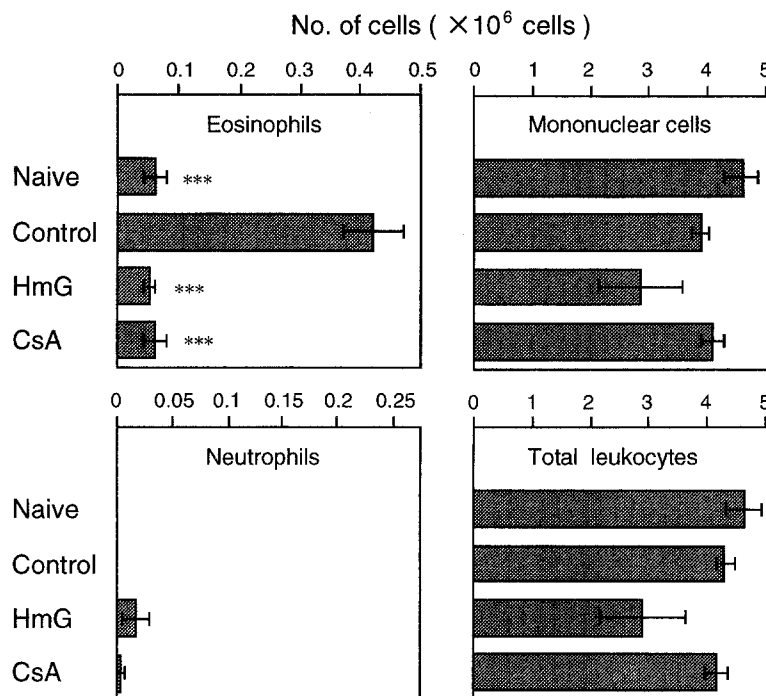


FIG. 1. Effect of HmG on number of eosinophils, neutrophils, and mononuclear cells in peritoneal cavity after intraperitoneal challenge with ragweed pollen in sensitized mice. Mice were sensitized subcutaneously with allergen on days 0, 1, 6, 8, and 14 (see Methods) and were challenged intraperitoneally with allergen on day 20. Peritoneal cells were obtained 24 hours after challenge. HmG at 150 mg/kg/day or saline solution was administered subcutaneously on days 0, 4, 7, 11, 14, and 18. Cyclosporin A at 100 mg/kg/day was administered subcutaneously on days 18, 19, and 20. Each value represents mean $\pm$ SEM of six animals (except for naive group in which $n = 3$). *** $p < 0.001$ as compared with control (saline-treated group). CsA, Cyclosporin A.

week for 6 weeks significantly improved the score of clinical symptoms such as coughing, wheezing, and the magnitude and/or frequency of asthma attacks (again compared with the effect of human γ -globulin injection).²⁸ A low incidence of side effects was observed (two of 60 patients [3.3%]).

HhG was developed by Parrot et al.^{29, 30} as a drug for restoring histaminopexy, the ability to block the biologic activity of histamine, in the sera of patients with allergy. In addition to this effect, HhG or a complex of histamine/rat γ -globulin has been reported to have inhibitory effects on antigen-induced histamine release (from human peripheral blood basophils or rat peritoneal mast cells, respectively).³¹⁻³³ However, the precise mechanism underlying the antiallergic action of HhG has yet to be completely elucidated. In view of the crucial role of eosinophils in allergic inflammation, we retrospectively analyzed clinical data on the use of HhG in cases of asthma, allergic rhinitis, and atopic dermatitis. We then realized that subcutaneous administration of HhG not only relieved clinical symptoms but also seemed to decrease the number of eosinophils in the nasal secretions or peripheral blood of allergic patients.³⁴⁻³⁶ Consequently, in this study we decided to explore the possibility that HhG actively inhibits the accumulation of eosinophils at inflammation sites. To this end we prepared a complex of histamine dihydrochloride (His)/

mouse γ -globulin (HmG), in the normal fashion for HmG, and we then examined whether HmG inhibited allergen-induced peritoneal eosinophil accumulation in mice. We show here for the first time that HmG (but not His or m γ G given separately) preferentially inhibits the allergen-induced peritoneal accumulation of eosinophils, but not of neutrophils, in mice. We discuss the relationship between the effect of HmG on eosinophil accumulation and the clinical efficacy of HhG in the treatment of allergic disease.

METHODS

Animals

Female BALB/c mice were purchased from Charles River Inc. (Kanagawa, Japan). Six- to seven-week-old mice were used for all experiments. This study was conducted in accordance with local and national guidelines governing the care and use of animals.

Chemicals and preparation of HmG

His, mouse γ -globulin (m γ C), serotonin, and mouse albumin were purchased from Sigma Chemical Co. (St Louis, Mo.). Glutamine, Cyclosporin A, and ragweed pollen extract (1:100, wt/vol) were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan; Sandoz Ltd., Basel, Switzerland; and Torii Pharmaceutical Company, Tokyo, Japan, respectively.

The "standard" HmG preparation was made as follows: 0.15 μ g of His was added to 12 mg of m γ C in saline solution and

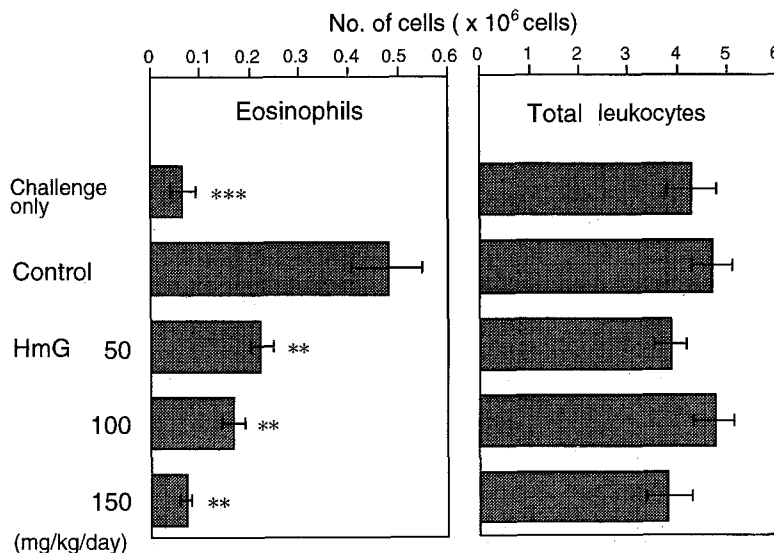


FIG. 2. Dose-dependent inhibition by HmG of eosinophil accumulation in mice. Procedures used for sensitization, challenge, and harvesting of peritoneal cells were as described in Fig. 1. HmG at 50 to 150 mg/kg/day or saline solution was administered subcutaneously on days 0, 4, 7, 11, 14, and 18. Each value represents mean $\pm$ SEM of 12 animals. ** p < 0.01, *** p < 0.001 as compared with control.

incubated for 2 hours at room temperature (23°C to 27°C). The resulting solution was stored at below -20°C until needed. Complexes of serotonin/m γ G, glutamine/m γ G, and His/mouse albumin were prepared by incubating the appropriate pair of agents in saline solution (at the same molar ratio as that used for the standard HmG solution) for 2 hours at room temperature. In experiments carried out to study the importance of incubation time, His and m γ G were incubated for 0, 15, 60, 120, or 240 minutes at room temperature; and the resulting complexes were administered subcutaneously at 150 mg/kg/day just after preparation. In experiments on the relative effectiveness of various combination ratios of His and m γ G, various amounts of His (1.5, 0.15, 0.015, or 0.0015 μ g) were incubated with 12 mg of m γ G in saline solution for 2 hours at room temperature. The resulting complexes were then stored at below -20°C until needed.

Allergen-induced cell accumulation in the peritoneal cavity in mice

This model was prepared according to the procedure of Kaneko et al.³⁷ BALB/c mice were immunized with a 1:1000 dilution of the commercially prepared ragweed pollen extract; 0.1 ml of this dilution was injected subcutaneously on days 0 and 1, and 0.2 ml was injected subcutaneously on days 6, 8, and 14. Then the mice were challenged intraperitoneally with 0.2 ml of the same dilution of the pollen extract on day 20. Peritoneal cells were harvested 0, 8, 24, or 48 hours after challenge with 4 ml of phosphate-buffered saline containing 1.0% fetal calf serum (Hyclone Laboratory Inc.) and 5 U/ml heparin. The total number of peritoneal cells from a given mouse was counted with a hemocytometer (model CC-110; Sysmex, Kobe, Japan). For this purpose, 50 μ l of the peritoneal cell suspension (5×10^5 cells/ml) was smeared on a microscope slide after centrifugation in a Cytospin 3 (Shandon, Jeol, Tokyo, Japan). A differential cell count was carried out under a microscope after fixation and staining with Giemsa dye (Wako Pure Chemical Industries, Ltd.).

Statistical analysis

Data are expressed as means $\pm$ SEM. Statistical significance was assessed by using an unpaired Student's t test.

RESULTS

Effect of HmG on allergic cell accumulation in mice

To investigate whether HmG inhibits allergic inflammatory cell accumulation in mice, its effect on allergen-induced peritoneal cell accumulation was examined. As shown in Fig. 1, a marked accumulation of eosinophils, but not of neutrophils or mononuclear cells, was observed in the control group after allergen challenge. HmG at 150 mg/kg/day markedly inhibited this eosinophil accumulation, reducing it to the level seen with 100 mg/kg/day Cyclosporin A. However, HmG had no significant effect on the number of neutrophils. Next, the dose-response relationship for this effect of HmG was examined by using the same system. At 50 to 150 mg/kg/day, HmG inhibited the eosinophil accumulation in a dose-dependent fashion (Fig. 2).

Time course of the effect of HmG on allergen-induced accumulation of eosinophils and neutrophils in mice

Mice were sensitized with the allergen and treated with HmG as indicated in Fig. 1. Peritoneal cells were then harvested 0, 8, 24, or 48 hours after challenge. As shown in Fig. 3, no significant accumulation of eosinophils was observed 8 hours after challenge in the control group in comparison with the 0-hour group (the "naive group"). In fact, the eosinophil accumulation reached a peak 24 hours after challenge, and the response gradually declined thereafter. When HmG was administered

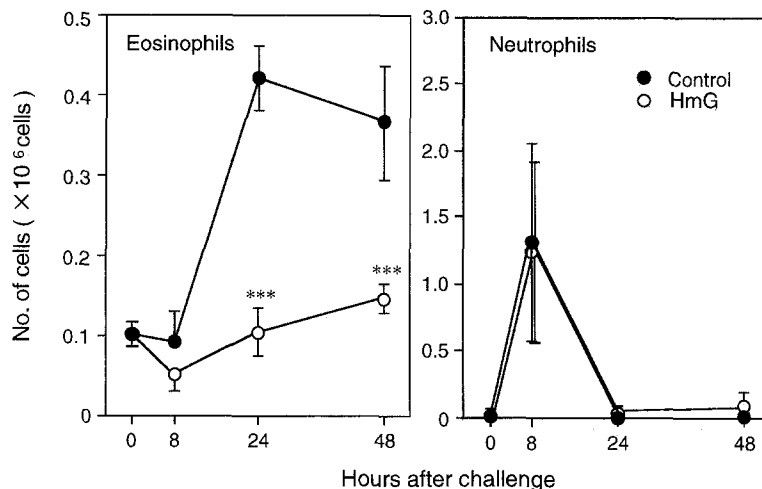


FIG. 3. Time course of effect of HmG on accumulation of eosinophils and neutrophils induced by allergen in mice. Procedures used for sensitization, challenge, and HmG administration were as described in Fig. 1. Peritoneal cells were harvested 0, 8, 24, 48 hours after challenge. Each value represents mean $\pm$ SEM of six animals (except for 0-hour group in which $n = 4$). *** $p < 0.001$ as compared with corresponding control.

subcutaneously during the 20-day sensitization period, at 150 mg/kg/day, it significantly inhibited the eosinophil accumulation 24 and 48 hours after challenge. In contrast, the accumulation of neutrophils reached a peak 8 hours after challenge, and their number then declined sharply, with only a small number being observed 0, 24, and 48 hours after challenge in the control group. Interestingly, HmG administration had no effect on the peak neutrophil accumulation.

Role of His and m γ G in the inhibition of eosinophil accumulation

The effect on eosinophil numbers was examined in the same mouse system when His or m γ G was given separately. Either His or m γ G at a dose equivalent to their doses in 150 mg/kg/day of HmG was administered subcutaneously six times during the 20-day sensitization period (by using the same protocol as before). As shown in Fig. 4, neither His nor m γ G had any inhibitory effect when given alone, although 150 mg/kg/day HmG did significantly inhibit the response (as before). Next, the effects of complexes other than HmG were examined. As shown in Fig. 5, complexes of serotonin/m γ G, glutamine/m γ G, and His/mouse albumin prepared in the same molar ratio as that used for the standard solution of HmG (0.15 μ g of His/12 mg of m γ G), did not exert significant inhibitory effects when administered subcutaneously at 150 mg/kg/day six times during the 20-day sensitization period. We next tried to clarify to what extent the effectiveness of HmG complexes depends on the ratio of His to m γ G within them. After various amounts of His (1.5, 0.15, 0.015, or 0.0015 μ g) had been incubated with 12 mg of m γ G in saline solution for 2 hours, 150 mg/kg/day of each complex was administered subcutaneously six times during the 20-day sensitization period. As shown in Fig. 6, a significant inhibition was

observed when the ratio was either 0.15 μ g of His/12 mg of m γ G or 0.015 μ g of His/12 mg of m γ G. It was also confirmed that complexes of serotonin/m γ G and glutamine/m γ G exerted no significant inhibition at either 0.1 or 10 times the standard molar ratio used for HmG (data not shown). The effects of incubation time were also examined. Immediately after His and m γ G had been incubated in saline solution for 0, 15, 60, 120, or 240 minutes, the resulting complexes were administered subcutaneously six times during the 20-day sensitization period. As shown in Fig. 7, no inhibitory effect was observed when the complex was administered immediately after mixing (time 0). However, when the complex was incubated for 15 minutes, it produced an inhibition of about 40%. Complexes that had been incubated for 60 or 120 minutes produced a plateau inhibition (70% to 80%).

DISCUSSION

In this study we demonstrated that HmG, a complex of His and m γ G, inhibits the allergen-induced peritoneal eosinophil accumulation in mice in a dose-dependent fashion. Interestingly, when given alone, neither His nor m γ G had any effect on such eosinophil accumulation. Administration of any one of a variety of doses of His or m γ G separately failed to uncover an inhibitory effect in the same system (unpublished data). Although His and γ -globulin have each been shown to regulate eosinophil functions through their specific receptors,³⁸⁻⁴⁰ little is known about the inhibition of eosinophil accumulation by a complex of His and γ -globulin. This study represents the first demonstration that HmG exerts an inhibitory effect on eosinophil accumulation.

Complexes of serotonin/m γ G, glutamine/m γ G, and His/mouse albumin prepared in the same molar ratio as that used for the standard HmG preparation did not

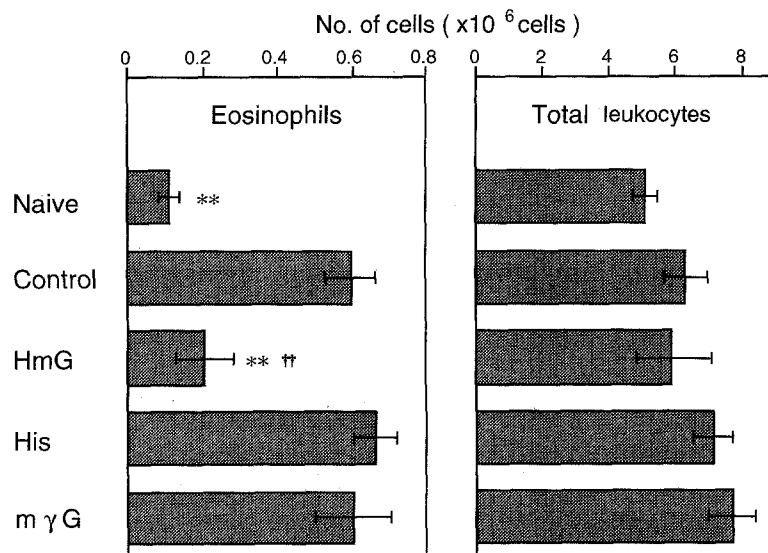


FIG. 4. Effect of HmG, His, and mγG on allergen-induced eosinophil accumulation. Procedures used for sensitization and challenge were as described in Fig. 1. HmG (150 mg/kg/day), His, or mγG (dose equivalent to their dose in 150 mg/kg/day HmG) was administered subcutaneously with the same protocol as in Fig. 1. Peritoneal cells were obtained 24 hours after challenge. Each value represents mean $\pm$ SE of six animals (except for naive group in which $n = 4$). ** $p < 0.01$ as compared with control. †† $p < 0.01$ as compared with mγG group.

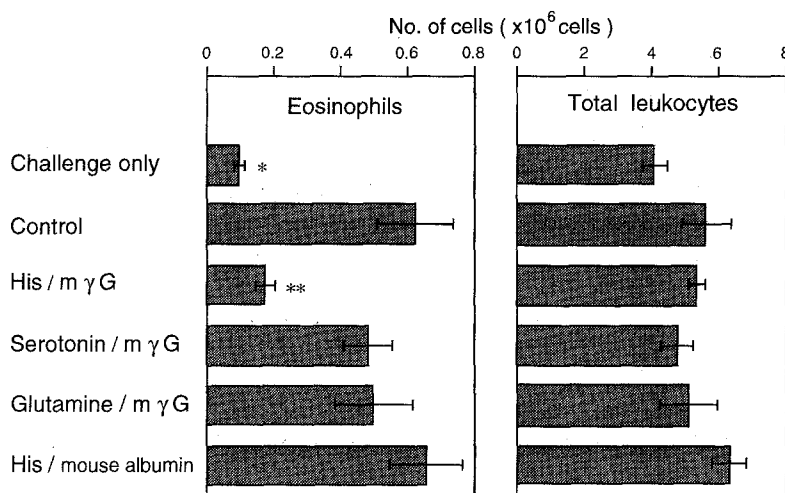


FIG. 5. Combination of His and mγG is required to induce an inhibitory effect on eosinophil accumulation. Procedures used for sensitization and challenge were as described in Fig. 1. Complexes of HmG, serotonin/mγG, glutamine/mγG, or His/mouse albumin (each at 150 mg/kg/day) or saline solution were administered as in Fig. 1. Peritoneal cells were harvested 24 hours after challenge. Each value represents mean $\pm$ SEM of 12 animals. ** $p < 0.01$ as compared with control.

exert any inhibitory effect. Furthermore, complexes of serotonin/mγG and glutamine/mγG at 0.1 or 10 times molar ratio produced no significant inhibition. Our study of incubation time indicated that 1 to 2 hours of incubation of His with mγG was needed to induce an optimum inhibitory effect. In addition, it was demonstrated that complexes involving ratios in the range of 0.015 μ g of His/12 mg of mγG to 0.15 μ g of His/12 mg of mγG exerted significant inhibitory effects. However,

as yet it is not clear why His and mγG need to be present together, or why certain combination ratios are needed to induce the inhibitory effect.

With regard to the identity of the active molecule or molecules in HmG, there seem to be four possibilities. First, His and mγG molecules that are not bound to each other act separately and synergistically to inhibit eosinophil accumulation. If this possibility were correct, the same inhibitory effect would be observed when the

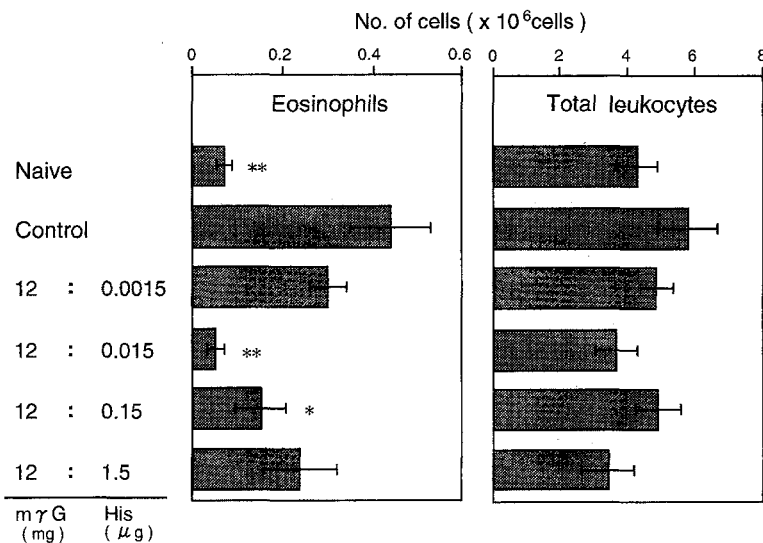


FIG. 6. Effect of mixtures of His and m γ G (in various ratios) on eosinophil accumulation. Procedures used for sensitization and challenge were as described in Fig. 1. Various complexes of His and m γ G (at 150 mg/kg/day) or saline solution were administered as in Fig. 1. Peritoneal cells were obtained 24 hours after challenge. Each value represents mean $\pm$ SEM of six animals. * p < 0.05, ** p < 0.01 as compared with control.

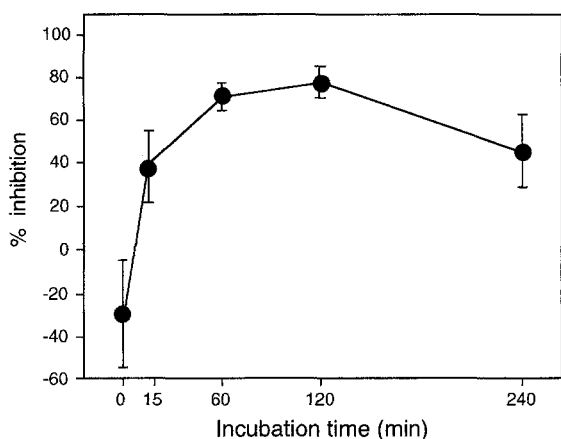


FIG. 7. Effect of time of incubation with His and m γ G on allergen-induced eosinophil accumulation. His and m γ G were incubated in saline solution for 0, 15, 60, 120, or 240 minutes. Complexes (at 150 mg/kg/day) or saline solution were administered subcutaneously immediately after preparation by using the protocol described in Fig. 1. Peritoneal cells were obtained 24 hours after challenge. Each value represents mean $\pm$ SEM of six animals.

complex is administered immediately after mixing (i.e., at incubation time 0). However, at incubation time 0, the preparation had no inhibitory effect. Therefore we can effectively exclude this possibility. Second, His-bound m γ G is the active molecule. At present, we do not even know whether His is bound to m γ G. Moreover, we do not know why, if it is the case, that certain optimum His/m γ G molar ratios (approximately 1:100 to 1:1000) are needed for the eosinophil inhibition to be apparent. An analysis in which an equilibrium dialysis method was used indicated that binding of His to m γ G was not detectable when 0.15 μ g of His was mixed with 12 mg of

m γ G in 1.0 ml of saline solution (unpublished data). However, approximately 10% of His was bound to 12 ng of m γ G or human γ -globulin in 1.0 ml of saline solution when a reduced amount of His (0.04 μ g) was used. These findings suggest that a very weak binding between His and m γ G occurs in saline solution. However, it is unclear at present whether His-bound m γ G is the active molecule. Third, the active molecule may be m γ G conformationally changed by His. A structural analysis of HmG done with a gel filtration method with high-pressure liquid chromatography and a nuclear magnetic resonance method is now being done. Fourth, impurities of m γ G or His may be the active principle or principles. The His (purity minimum, 99%), m γ G (purity minimum, 95%), and mouse albumin (fraction V powder) used in this study contained little or none of the biologically active substance, endotoxin (no detection for His, but 0.1 to 0.7 ng/mg for m γ G and 0.6 ng/mg for mouse albumin). If endotoxin were the active molecule, administration of His or m γ G alone would produce an inhibitory effect on eosinophil accumulation. However, when given separately, neither His nor m γ G at a dose equivalent to their dose in 150 mg/kg/day of HmG produced a significant inhibition, despite the fact that a mixture of His/m γ G (150 mg/kg/day) markedly inhibited eosinophil accumulation. Furthermore, a complex of His/mouse albumin at 150 mg/kg/day did not significantly inhibit eosinophil accumulation. Therefore even though endotoxin may be present in HmG, it seems not to be the active ingredient. For similar reasons, it is unlikely that other impurities of m γ G or His are the active molecule or molecules.

This time-course study of the allergen-induced cell accumulation further demonstrated that HmG inhibited the accumulation of eosinophils but not of neutrophils. In addition, we have found that HmG at 150 mg/kg/day

exerts no effect on lipopolysaccharide-induced neutrophil accumulation in mice (unpublished data). Thus it seems likely that HmG has no significant effect on neutrophil accumulation in mice. Recent studies have suggested that eosinophils, but not neutrophils, are selectively infiltrated from blood to inflammation site through (1) the action of eosinophil chemoattractants, including IL-5 and eotaxin and (2) the interaction between the effect of very late activation antigen-4 on eosinophils and that of vascular cell adhesion molecule-1 on endothelial cells.^{12, 13, 41, 42} Therefore we need to consider the following possibilities in our attempts to elucidate the mechanism underlying the inhibitory effect of HmG: (1) HmG inhibits the production, release, or both of eosinophil chemoattractants such as IL-5 and eotaxin from T_{H2}-type helper T cells or other cells; (2) HmG preferentially suppresses chemotaxis in eosinophils; (3) HmG induces apoptosis in eosinophils but not in neutrophils; (4) HmG selectively inhibits the expression of very late antigen-4, vascular cell adhesion molecule-1, or both; and (5) HmG preferentially suppresses the maturation of eosinophils from bone marrow cells. With respect to IL-5 production, we have demonstrated that HhG does not inhibit allergen-induced IL-5 release from peripheral blood mononuclear cells in patients with atopic asthma (unpublished data). On this basis, it seems unlikely that HmG inhibits IL-5 release from T_{H2}-type helper T cells in this mouse model. Studies to explore the possibilities listed above are in progress at cellular and molecular levels in our laboratories.

At 150 mg/kg/day, HmG also inhibited the eosinophil accumulation when administered subcutaneously on 3 consecutive days up to and including the challenge day, even though these were days on which the mice had been sensitized (unpublished data). This finding in mice suggests that HhG may prove to actively inhibit allergen-induced eosinophil accumulation in patients with allergy (who are already sensitized). Interestingly, in double-blind clinical studies of bronchial asthma, allergic rhinitis, and atopic dermatitis, HhG injected subcutaneously twice per week for 6 weeks caused a significant decrease in the clinical symptoms over and above any effect seen with human γ -globulin alone.^{26-28, 43} An apparently similar finding in this study was that HmG administered subcutaneously six times (twice per week) to mice during the 20-day sensitization period significantly inhibited the eosinophil accumulation, whereas m γ G alone did not. The similarity between these two sets of data strongly suggests that HhG may exert its therapeutic effects mainly through the active suppression of eosinophil accumulation.

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