

The House Dust Mite Allergen, *Dermatophagoides pteronyssinus*, Promotes Type 2 Responses by Modulating the Balance Between IL-4 and IFN- γ ¹

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A common property of allergens is their potential to generate type 2 cytokine responses. To understand the mechanisms involved in this phenomenon, we have evaluated the polarizing potential of a major allergen, *Dermatophagoides pteronyssinus* 1 (Der p 1), in an heterologous immunization system using the glutathione S-transferase of the parasite *Schistosoma mansoni* (Sm28-GST) as immunogen. In previous studies, we showed that immunization with the Sm28-GST emulsified in CFA induced a nonpolarized immune response. In contrast, when alum was used as adjuvant, a type 2 immune response was induced against Sm28-GST. Using this experimental model, we examined whether the administration of Der p 1 together with Sm28-GST influenced the nonpolarized and/or the Th2 profiles induced by the CFA or the alum immunization, respectively. Our results showed that the introduction of Der p 1 in the CFA immunization protocol was associated with diminished anti-Sm28-GST IgG2a Ab titers, reduced IFN- γ mRNA expression, and frequency of IFN- γ -producing cells. In contrast, the introduction of Der p 1 in the alum protocol did not affect IL-4 or Ig isotype responses. The effect of Der p 1 was specific, since coimmunization with tetanus toxin fragment C did not affect the profile of the response against Sm28-GST. Furthermore, inactivation of Der p 1 reduced its ability to modify the immune response profile, suggesting that its protease activity played an important role in deviating the immune response. Our results suggest that the Der p 1 has the ability to modify the profile of an immune response by modulating the balance between the polarizing cytokines IL-4 and IFN- γ . *The Journal of Immunology*, 1998, 160: 2456–2462.

Allergens are a heterogeneous class of substances (1) involved in the pathogenesis of anaphylactic type reactions in humans and in animals. Immune responses against allergens are characterized mainly by specific IgE Ab production (2, 3) and eosinophilia (4). Since IgE production (5–7) and eosinophil maturation and migration (8, 9) are positively controlled by the type 2 cytokines IL-4 and IL-5, respectively, allergens are thought to be able to induce type 2 cytokine responses. This is supported by studies showing that allergen-specific T cell clones from allergic patients produce type 2 cytokines when stimulated in vitro (10–12). In addition, these patients do not show a generalized predilection to mount type 2 responses, since their response against nonallergens is usually nonpolarized (10, 11). However, since allergen-specific T cells have been obtained in these studies from already sensitized individuals, the possibility that factors other than the allergen itself are responsible for the initiation of a type 2 response cannot be excluded.

The observation that many allergens, especially aeroallergens, and parasite Ags possess proteolytic activities (13–15), has raised

the possibility that this common property may be essential for their ability to generate IgE and Th2 cytokine responses. Some direct evidence showing that the proteolytic enzyme papain stimulates IL-4 gene expression has been provided recently in support of this hypothesis (16). Also, the possibility that the major allergen of the house dust mite, *Dermatophagoides pteronyssinus* (Der p 1),³ may modulate IgE expression through its ability to cleave membrane CD23 to its soluble form has been proposed in recent studies (17, 18). However, determination of whether allergens possess Th2-polarizing capacity in vivo is complicated by the fact that immunization in adjuvants is often needed to elicit an immune response against allergens, and adjuvants themselves may influence the type of the immune response generated (19, 20). In previous studies, it has been shown that a bystander immune response could influence the type of the response against an irrelevant Ag (21–23). We reasoned, therefore, that it would be possible to test the polarizing capacity of an allergen in an heterologous immunization system. To do that, we examined whether coadministration of Der p 1 influenced the immune response against an irrelevant Ag, for instance, the *Schistosoma mansoni* glutathione S-transferase (Sm28-GST). In a previous study, we showed that immunization with Sm28-GST in CFA induced a nonpolarized immune response, in contrast to immunization in aluminum hydroxide (AH), which induced a type 2 immune response against Sm28-GST (19). In this study, we show that administration of Der p 1 together with Sm28-GST in CFA diminished IFN- γ production and the specific anti-Sm28-GST IgG2a Ab response. In addition, this property of Der p

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³ Abbreviations used in this paper: Der p 1, *Dermatophagoides pteronyssinus*; Sm28-GST, *Schistosoma mansoni* glutathione S-transferase; AH, aluminum hydroxide; TtC, tetanus toxoid fragment c; HPRT, hypoxanthine phosphoribosyltransferase; ELISPOT, enzyme-linked immunospot.

1 seems to be, in part, dependent on its protease activity, since inactivated Der p 1 presented reduced capacity to modulate immune responses against Sm28-GST.

Materials and Methods

Mice

Six- to eight-week-old female BALB/c mice were purchased from IFFA-CREDO (L'Arbresle, France). Mice were maintained under pathogen-free conditions throughout the study.

Ags

Escherichia coli expressing recombinant Sm28-GST was cultured in LB medium, and recombinant protein was purified as previously described (24). Recombinant tetanus toxoid fragment c (TTC) was purchased from Boehringer Mannheim (Meylan, France). Der p 1 was isolated by affinity chromatography from fecally enriched spent mite growth medium as previously described (25). The material eluted from the mAb affinity column was further purified by chromatofocusing and the protein identified by using an insoluble protease substrate (Azocoll; Calbiochem, Sydney, Australia).

Enzymatic activity of Der p 1 was inhibited either by heating (95°C, 1 h) (see Ref. 26), or by adding the cysteine protease inhibitor E-64 (trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane; Sigma, St. Quentin, France), at 7 mol/mol of Der p 1.

Immunizations

Mice were injected s.c. in the base of the tail with 20 µg of recombinant Sm28-GST adsorbed on AH (final concentration 0, 6% w/v) or emulsified (v/v) in CFA (Difco Laboratories, Detroit, MI). In most experiments, 20 µg of Der p 1 were injected/mouse, unless otherwise mentioned. In coimmunization experiments Sm28-GST and Der p 1 were present in the same immunization mixture.

Ab measurement by ELISA

IgG1 and IgG2a Ab response to Sm28-GST were measured by ELISA as previously described (19). Briefly, 96-well plates (Immulon II; Dynatech, Denkendorf, Germany) were coated overnight with Sm28-GST (0.25 µg/well) in 0.1 M carbonate buffer, pH 9.6, at 4°C. After washing, serial dilutions of sera were added, in PBS/0.1% Tween 20/0.5% gelatin for 2 h at room temperature. Plates were then washed and biotin-conjugated goat anti-mouse IgG1 or IgG2a Abs (Southern Biotechnology Associates, Birmingham, AL) diluted at 1/45,000 were added for 1 h at room temperature. After further washes, peroxidase-coupled streptavidin (Jackson ImmunoResearch, West Grove, PA) was added for 30 min. After extensive washing, 50 µl of substrate solution (1 mg/ml orthophenylene diamine, Sigma, St. Louis, MO) was added to the wells. The color reaction was terminated by addition of 50 µl of 3 N HCl, and OD was measured at 492 nm.

Reverse transcription-polymerase chain reaction (RT-PCR)

Spleens and inguinal lymph nodes were removed from mice after cervical dislocation and stored immediately in RNazol (Bioprobe, Montreuil sur Bois, France). RNA was extracted according to the manufacturer's instructions. Levels of cytokine mRNA were assessed by a semiquantitative RT-PCR, as previously described (27). RNA was reverse transcribed using an RNase H-Reverse Transcriptase (Superscript II; Life Technologies, Gaithersburg, MD). Briefly, 2.5 µg of RNA were heated 10 min at 70°C, then mixed with 1 mM of each dNTP (Pharmacia Biotech., Orsay, France), 0.4 mM of random hexamers (pd(N)6, Pharmacia), 40 U of RNase inhibitor (Pharmacia), and 200 IU of reverse transcriptase in reaction buffer supplied by the manufacturer. The mixture was incubated for 1 h at 37°C and 5 min at 95°C, and then frozen at -20°C until PCR analysis. PCR was performed on the resultant cDNA to amplify HPRT, IL-4, IL-10, and IFN-γ. Reaction mixtures (25 µl) contained 100 ng cDNA, 0.1 mM of each dNTP, 8 to 80 pmol of each primer, 1.5 to 3.5 mM of MgCl₂ (depending on the cytokine) (see Ref. 19) and 1.25 U of Taq DNA polymerase (Promega, Madison, WI). Amplifications were performed as follows: 94°C, 3 min and 40 s; followed by 21 to 34 amplification cycles (94°C, 20 s; 54°C, 30 s; 72°C, 45 s) determined so that a linear relation existed between the amount of input cDNA and the final PCR product. Resulting cDNA was Southern blotted and hybridized with ³²P-labeled probes, and radioactive signals were quantified with a PhosphorImager (Molecular Dynamics, Evry, France). To compare different samples, the number of amplification cycles that resulted in a linear correlation between radioactive signal

intensity and input cDNA was defined for each cytokine, as previously described (19, 27).

To correct for sample variations, cDNA for the housekeeping gene HPRT was initially quantified, and a correction factor was applied to each sample. Signal intensities were then compared with those obtained for samples from control mice, and results were expressed as index of stimulation comparing to the control, arbitrarily considered as 1.

Lymphocyte cultures

Spleens and inguinal lymph nodes were isolated from mice on day 7 after immunization, and cells cultured in a concentration of 4 × 10⁶ cells/ml of RPMI 1640 supplemented with 10% FCS (Boehringer Mannheim), 2 mM L-glutamine, and 25 mM 2-ME. Cells were stimulated in triplicate with either 20 µg of Sm28-GST, 20 µg of conalbumin (as a negative control), or 5 µg of Con A (Sigma) per ml. Supernatants from 72-h cultures were collected and frozen until IL-4 and IFN-γ were determined.

Lymphokine measurements by ELISA

Concentrations of IL-2, IL-4 and IFN-γ were determined by ELISA. Clones producing mAb for detecting IL-2 (JES-1A12 and JES-5H4), IL-4 (BVD-4 and BVD-6), and IFN-γ (R46A2 and AN-18) by ELISA (28) were kindly provided by Dr. A. O'Garra (DNAX). Ninety-six-well plates (Nunc, Naperville, IL) were coated 2 h at 37°C with capture Abs (50 to 100 ng/well) in either carbonate buffer or PBS. After washing with PBS/0.1% Tween 20, supernatants were diluted 1/2 and added overnight at 4°C. After washing, 25 to 50 ng of biotin-conjugated Ab were added per well for 90 min at room temperature. After additional washing, peroxidase-labeled streptavidin was added for 1 h at room temperature. This was followed by washing and substrate addition. Supernatants were compared with standard curves, generated by serial dilutions of rIL-2, IL-4, and IFN-γ (PharMingen, San Diego, CA). The sensitivity of these ELISA ranged from 0.3 to 40 ng/ml for IL-2, 1 to 500 pg/ml for IL-4, and from 60 to 16,000 pg/ml for IFN-γ.

ELISPOT assays

IL-4- and IFN-γ-secreting cells were evaluated by ELISPOT assay, as previously described (29). Briefly, 96-well plates (Immulon II) were coated overnight with capture Abs for IL-4 (BVD-4) or IFN-γ (R46A2) (500 ng/well) in PBS at 4°C. Single-cell suspensions from spleens of immunized mice, or from 3-day cultures with Sm28-GST or conalbumin, were serially diluted (first concentration of 0.5 × 10⁶ cells/well) and incubated in coated plates overnight at 37°C. After extensive washes in PBS and PBS-Tween 0.1%, 200 ng/well of biotin-conjugated Abs for IL-4 (BVD-6) or IFN-γ (XMG-6) were added for 2 h at 37°C. After additional washing, alkaline-phosphatase-labeled streptavidin (Jackson ImmunoResearch) was added for 1 h at 37°C. Then, the substrate solution (1 mg/ml of 5-bromo-4-chloro-3-indolylphosphate (Sigma) in a 0.1 M 2-amino-2-methyl-1 propanol (Sigma)) was mixed with 0.6% Sea-plaque agarose (FMC Bioproducts, Rockland, ME) and 100 µl of this solution were added per well. Spots were counted using a dissecting microscope.

Statistical analysis

Because normality of data distribution was not demonstrated, nonparametric tests (Kruskal-Wallis and Mann-Whitney) were used to evaluate for statistical differences in Ig production and cytokine expression among different groups of mice.

Results

Coadministration of Der p 1 modifies the isotypic response against Sm28-GST

In a previous study (19), we demonstrated a strong correlation between specific IgG1 and IgG2a anti-Sm28-GST Abs and IL-4 and IFN-γ expression, respectively, in mice immunized against Sm28-GST in different adjuvant formulations. Thus, mice immunized with Sm28-GST in CFA made a nonpolarized response characterized by both IL-4 and IFN-γ production and specific IgG1 and IgG2a Abs. When Sm28-GST was administered in alum, a type 2 response developed with high IL-4 and IgG1 anti-Sm28-GST titers. In preliminary experiments, we also observed that, similarly to Sm28-GST, isotypic profiles against Der p 1 were influenced by the adjuvant used (data not shown). However, when the CFA was used as adjuvant, the anti-Der p 1 IgG1/IgG2a ratio was higher

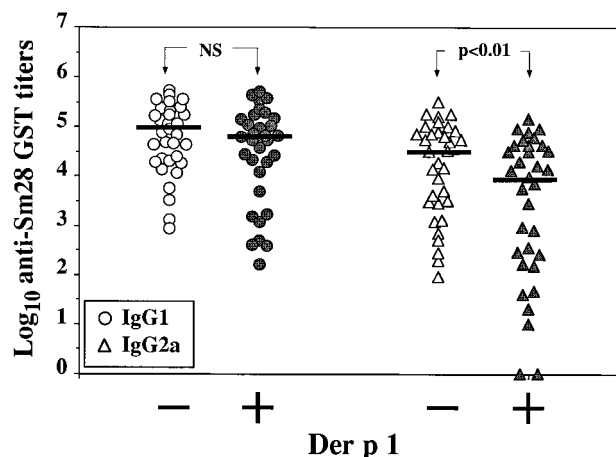


FIGURE 1. Anti-Sm28-GST IgG1 (circles) and IgG2a (triangles) responses induced by immunization with Sm28-GST in CFA in the presence (shaded symbols) or absence (open symbols) of Der p 1. Mice were immunized s.c. with a single dose of Sm28-GST (20 μ g/mouse; in CFA) alone or in the presence of 20 μ g of Der p 1. Sera were collected 28 days after immunizations, and anti-Sm28-GST IgG1 and IgG2a levels were measured by ELISA. Results from six independent experiments each comprising 5 to 10 mice are shown in this graph. Each symbol represents the Ig titer of an individual mouse. The bars show the median.

than the anti-Sm28-GST IgG1/IgG2a ratio, suggesting that the immune response against Der p 1 presents a more Th2-like profile. Based on these results, we examined whether affinity-purified Der p 1 would influence isotypic profiles against coinjected Sm28-GST in CFA. In initial experiments, similar isotype profiles were observed in 14-, 21-, and 28-day sera from single-dose immunized mice. Because on day 28 higher anti-Sm28-GST titers were observed, only this time point was examined in subsequent experiments. In six independent experiments, average and median IgG2a anti-Sm28-GST titers strongly diminished in Der p 1-coimmunized mice; IgG1 titers also diminished but to a lesser degree (Fig. 1). For statistical analysis we first determined, using the Kruskal-Wallis nonparametric rank test, that inter-experiment variation was not significant. Based on this, results from all the experiments were grouped and analyzed together. Comparison of IgG2a anti-Sm28-GST titers in mice immunized with both Sm28-GST and Der p 1 or with only Sm28-GST showed that the former had significantly lower titers ($p < 0.01$; Mann-Whitney test). In contrast, IgG1 titers were not statistically different in the two groups of mice. Also, the IgG1/IgG2a ratio significantly increased ($p < 0.02$) in Sm28-GST/Der p 1-immunized mice (data not shown). To eliminate the possibility that the decrease in the anti-Sm28-GST titers was due to antigenic competition, control experiments were performed in which mice were immunized with Sm28-GST plus TTc. Analysis of sera, 28 days after immunization, showed that IgG1 and IgG2a anti-Sm28-GST titers were similar in single- and coimmunized animals (data not shown). To further confirm that antigenic competition was not the reason of the diminished anti-Sm28-GST titers in Der p 1-coimmunized animals, mice were immunized with 10 or 40 μ g of Der p 1 plus Sm28-GST, or with the latter alone, in alum (Th2 protocol). With this protocol, Der p 1 had a dose-dependent positive effect on IgG1 titers, while IgG2a anti-Sm28-GST titers remained at baseline levels in all groups of mice (data not shown). This result was confirmed in four independent experiments, in which IgG1 titers increased from 1.3- to 3.4-fold in Der p 1-coimmunized mice. However, no statistically significant difference was demonstrated. A dose effect of Der p 1 was also observed in the CFA protocol. As shown in Figure 2, 10 μ g

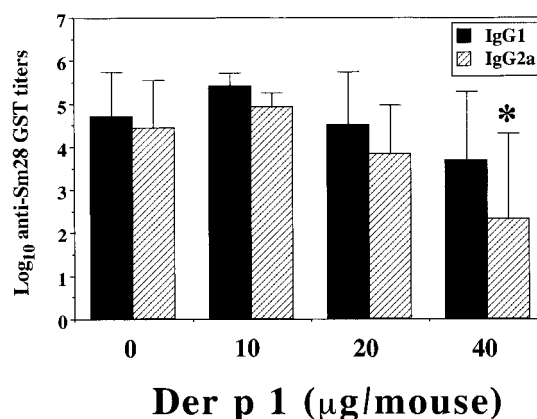


FIGURE 2. Dose-dependent influence of Der p 1 on the anti-Sm28-GST IgG1 (solid bars) and IgG2a (shaded bars) responses induced by immunization with Sm28-GST in CFA. Mice (10/group) were immunized once s.c. with Sm28-GST (20 μ g/mouse; in CFA) alone, or plus different doses of Der p 1. Sera were collected 28 days after immunizations, and anti-Sm28-GST IgG1 and IgG2a levels were measured by ELISA. Results (mean $\pm$ SE) are expressed as logarithm of sera titers. One representative experiment out of two is shown. Statistical differences ($p < 0.05$) between Sm28-GST- and Sm28-GST/Der p 1-immunized mice are denoted with an asterisk (*).

of Der p 1 had no effect on IgG titers; with 20 μ g a 2.6-fold decrease of IgG2a was observed, but no statistically significant difference was demonstrated. In contrast, with 40 μ g of Der p 1 both IgG1 and IgG2a diminished but only the latter showed a statistically significant decrease ($p < 0.05$). In contrast to the effect that the presence of Der p 1 had on Sm28-GST isotypic profiles, no changes on Der p 1 Ab profiles were observed in coimmunized mice (data not shown).

These results suggest that Der p 1 has the potential to inhibit Th1-associated isotype production against an irrelevant Ag.

Der p 1 influences cytokine mRNA expression in vivo

Since IgG2a production is promoted by IFN- γ and inhibited by IL-4 (30), these results prompted us to examine whether Der p 1 coimmunization would influence the expression of these cytokines. As expected from a previous study (19), immunization of mice with Sm28-GST in CFA induced increased IL-4 and IFN- γ mRNA expression in draining lymph nodes 7 days after immunizations (Fig. 3). Similar IL-4 mRNA levels were expressed in Sm28-GST/Der p 1 and in mice immunized with Sm28-GST alone. At the same time, IFN- γ mRNA expression decreased to almost baseline levels in Sm28-GST/Der p 1 immunized mice. This decrease was statistically significant ($p < 0.02$) as demonstrated with the Mann-Whitney test. Similar results were observed on day 10 after immunization (data not shown). Because IL-10 has been shown to inhibit IFN- γ production (31, 32), the expression of mRNA coding for this cytokine was also examined in the same mice. In fact, IL-10 mRNA expression did not increase significantly in either group of mice on day 7 (Fig. 3) or at any other time point examined (4, 8, 24, 48, and 96 h, and 10 days) (data not shown).

To further analyze the effect of Der p 1 on IL-4 and IFN- γ mRNA expression, kinetic experiments were performed. As shown in Figure 4, IL-4 mRNA expression increased similarly, as early as 4 h after immunization, in both groups of mice. At later time points, however (24 and 48 h), the increase in IL-4 mRNA in Der p 1 coimmunized mice was larger than in the group immunized with Sm28-GST alone. Statistical analysis of three independent experiments showed that the difference observed at 48 h was

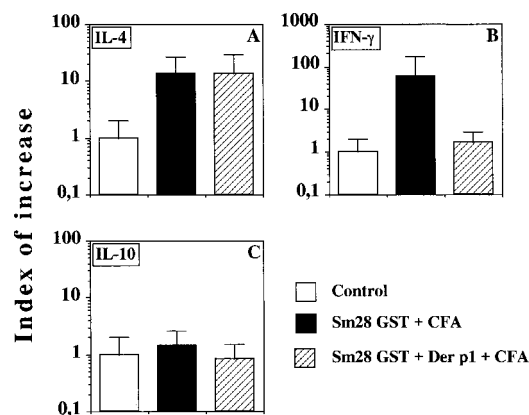


FIGURE 3. Levels of IL-4 (A), IFN- γ (B), and IL-10 (C) mRNA after immunizations with Sm28-GST in the presence or absence of Der p 1. Mice (five/group) were either immunized s.c. with Sm28-GST (20 μ g/mouse; in CFA) alone (solid bars), or in the presence of 20 μ g of Der p 1 (shaded bars). Seven days after immunizations, mice were killed and RNA from inguinal lymph nodes was assessed by semiquantitative RT-PCR for cytokine mRNA expression. Results are expressed as stimulation indices, relative to control mice injected with 0.9% NaCl (open bars), (arbitrarily considered as 1; see *Materials and Methods*). Mean $\pm$ SE of three experiments are shown.

highly significant ($p < 0.01$). At the 96- and 168-h time points, similar levels of IL-4 mRNA were observed. IFN- γ mRNA expression was similar in both groups of mice until the 96-h time point. At 168 h, however, a highly significant difference ($p < 0.01$) in IFN- γ mRNA expression was observed between the Sm28-GST and the Sm28-GST/Der p 1 immunized mice. IFN- γ mRNA increased 10-fold in the former group and only 2-fold in the latter. These results show that Der p 1 exerts a positive effect on early IL-4 mRNA and a negative effect on late IFN- γ expression. Without excluding other possibilities, this result suggests that the diminution of Th1-associated response in Der p 1-coimmunized mice could be the result of the early increased production of IL-4, which inhibits later IFN- γ expression.

IL-4 and IFN- γ production in single- and coimmunized mice

Experiments were performed to examine whether the Der p 1 effects on IFN- γ and IL-4 mRNA expression resulted in similar changes in the production of these cytokines. To this end we analyzed the frequency of IL-4- and IFN- γ -producing spleen cells, in mice immunized with Sm28-GST or with Sm28-GST plus Der p 1 in CFA or in AH ex vivo, by ELISPOT assay. Splenocytes of Sm28-GST/Der p 1-immunized mice had a significantly lower frequency ($p < 0.05$) of IFN- γ production as compared with their counterparts that received only Sm28-GST in both the alum and the CFA immunization protocol (Fig. 5). In fact, the frequency of IFN- γ -producing cells dropped even lower than that of the control mice that received only normal saline. In contrast, the frequency of IL-4 producing cells was not affected by the presence of Der p 1. To determine whether the Ag-driven cytokine production was also diminished in Sm28-GST/Der p 1-immunized mice, spleen cells from 7-day immunized mice were stimulated in vitro with Sm28-GST for 3 days, and the frequency of IL-4- and IFN- γ -producing cells was determined by ELISPOT. We found that Sm28-GST-stimulated cells from both Sm28-GST- and Sm28-GST/Der p 1-immunized mice showed similar frequencies of IL-4 production (data not shown). In contrast, the frequency of IFN- γ -producing cells in the latter group decreased approximately threefold in comparison with that observed in the Sm28-GST-immunized group.

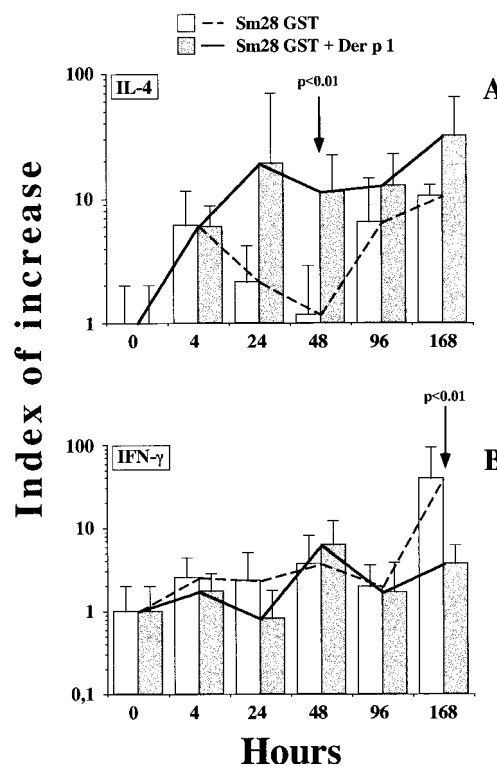


FIGURE 4. Kinetics of IL-4 (A) and IFN- γ (B) mRNA expression after immunization with Sm28-GST in the presence or absence of Der p 1. Mice (five/group) were immunized s.c. with Sm28-GST (20 μ g/mouse; in CFA) alone (open bars), or in the presence of 20 μ g of Der p 1 (shaded bars). At the indicated time points mice were killed and RNA from inguinal lymph nodes was assessed by semiquantitative RT-PCR for cytokine mRNA expression. Results of three independent experiments are combined and expressed as stimulation indices, as compared with RNA values from control mice that had been injected with 0.9% NaCl (see *Materials and Methods*). Statistical differences between Sm28-GST- and Sm28-GST/Der p 1-immunized mice at the indicated time points are noted with arrows.

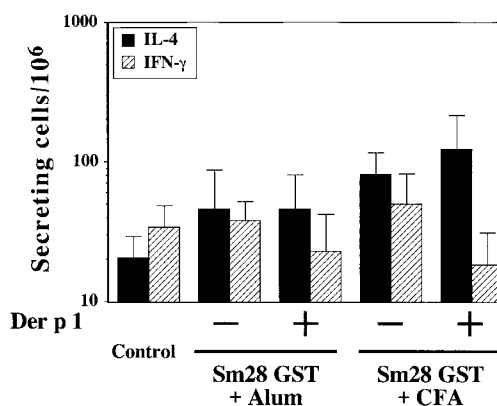


FIGURE 5. Ex vivo frequency analysis of IL-4-producing cells (solid bars) and IFN- γ -producing cells (shaded bars) after immunizations with Sm28-GST in the presence or absence of Der p 1. Mice (three or five/group) were immunized once s.c. with Sm28-GST (20 μ g/mouse; in CFA or alum) alone, or in the presence of 20 μ g of Der p 1. Seven days after immunizations, mice were killed, spleen cells from individual mice (first experiment) or from each group were pooled (second experiment), and serial dilutions of the cells deposited on anti-IL-4- or anti-IFN- γ -coated wells to determine the frequency of cytokine-producing cells.

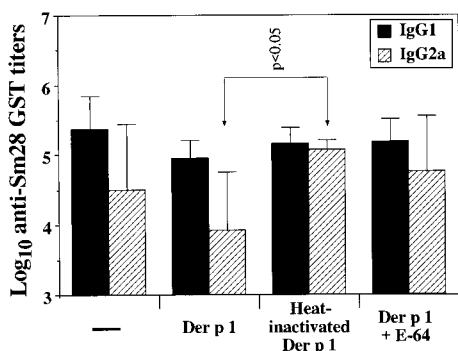


FIGURE 6. Effect of inactivation of Der p 1's enzymatic activity on anti-Sm28-GST IgG1 and IgG2a responses. Mice (five/group) were single-dose immunized s.c. with Sm28-GST (20 μ g/mouse; in CFA) alone or in the presence of 20 μ g of Der p 1 in a native form or inactivated by heat or with the E-64. Sera were collected 28 days after immunizations, and anti-Sm28-GST IgG1 (solid bars) and IgG2a (shaded bars) levels were measured by ELISA. Results (mean $\pm$ SE) are expressed as logarithm of sera titers. (One representative experiment of two is shown.)

Collectively, these results suggest that Der p 1 diminished the frequency of IFN- γ but not that of IL-4-producing cells.

The immunomodulatory effects of Der p 1 are probably due to its protease activity

To determine whether the protease activity of Der p 1 is necessary for its immunomodulatory effect, experiments with the specific cysteine protease inhibitor E-64 or heat treatment were performed. In the first experiment (Fig. 6), we examined whether inactivated Der p 1 modified isotypic responses against the Sm28-GST in coimmunization experiments. In this experiment, Sm28-GST-immunized mice showed a relatively high specific anti-Sm28-GST IgG1/IgG2a ratio of 4.2. In active Der p 1 coimmunized mice, both IgG1 and IgG2a titers diminished, with the latter showing a slightly larger decrease. When heat-inactivated Der p 1 was used, both IgG1 and IgG2a titers increased, but only the IgG2a titers demonstrated a statistically significant increase when compared with those of mice that received native Der p 1 ($p < 0.05$). A similar increase was observed in the group of mice that received Sm28-GST/Der p 1 in combination with the specific cysteine protease inhibitor E-64. In addition, Ab titers in control mice that received Sm28-GST plus E64 were similar to those in mice immunized with Sm28-GST alone, excluding the possibility that E-64, by itself, affects the Ab response (data not shown). Next, we examined the effect of heat-inactivated Der p 1 on IFN- γ expression. Mice immunized with Sm28-GST alone or with Sm28-GST plus native Der p 1 or heat-inactivated Der p 1 were killed 7 days later, and spleen cells were cultured for 3 days with Sm28-GST or conalbumin in control cultures. Cells from Der p 1-coimmunized mice produced threefold less IFN- γ than cells from mice immunized only with Sm28-GST (Fig. 7). Cells from mice coimmunized with heat-inactivated Der p 1 showed only a 40% decrease in their capacity to produce IFN- γ . Statistical analysis showed that significant differences existed between all groups of mice. These results were confirmed when the frequency of cells producing IFN- γ was examined by ELISPOT assay. Der p 1 reduced the frequency of IFN- γ -producing cells by 3.5-fold, in contrast to heat-inactivated Der p 1 in which reduction was only 1.8-fold (data not shown). Thus, these results strongly suggest that the immunomodulatory effects of Der p 1 are, in part, due to its protease activity.

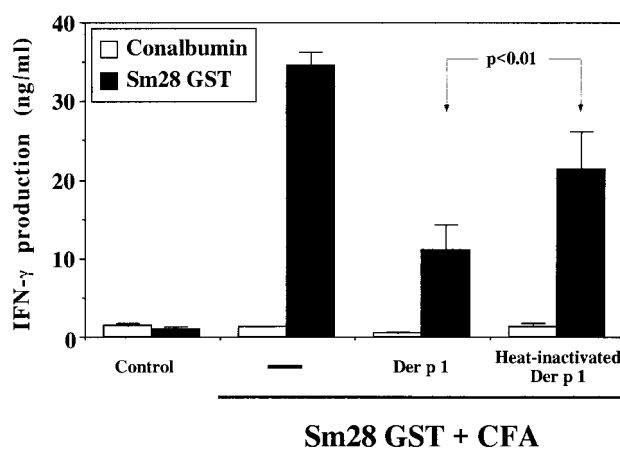


FIGURE 7. Effect of Der p 1 coimmunization on IFN- γ production after Ag-specific stimulation in vitro. Mice (three/group) were either immunized s.c. with Sm28-GST (20 μ g/mouse; in CFA) alone or in the presence of 20 μ g of native or heat-inactivated Der p 1. Seven days after immunization, mice were killed and spleen cells were stimulated in triplicate in vitro (4×10^6 cells/ml) with either Sm28-GST (20 μ g/ml, solid bars) or conalbumin (20 μ g/ml, open bars) as an irrelevant protein. Supernatants were assessed for IL-4 and for IFN- γ by ELISA. Results are expressed as the mean of triplicates ($\pm$ SE).

Discussion

Currently, it is not known why the immune response to allergens polarize to type 2. Moreover, no direct evidence exists demonstrating that allergens are the primary factor causing type 2 cytokine deviation of the immune response. Since, in nature, allergens enter the organism in association with a multitude of substances, the possibility exists that some other factors associated with the allergen drive the response to type 2.

In the present study, this question was studied by comparing the response induced against an irrelevant Ag, the Sm28-GST, in the presence or absence of the major domestic allergen, Der p 1, in the immunization mixture. We have observed that, when both Ags emulsified in CFA were injected in mice, Der p 1 was able to substantially decrease the Th1-associated IgG2a response against Sm28-GST. In addition, analysis of IFN- γ and IL-4 showed that Der p 1 induced an imbalance in the expression of these cytokines. In particular, the expression of mRNA coding for IFN- γ as well as the frequency of IFN- γ -producing cells was inhibited. It is of interest that when alum was used as adjuvant, the frequency of IFN- γ -producing cells in Der p 1/Sm28-GST-immunized mice dropped below the levels observed in control mice. Since IL-4 and IFN- γ are antagonistic cytokines that reciprocally inhibit each other's expression (33), one would expect that decreased IFN- γ expression would result in increased IL-4 expression and IgG1 production. To our surprise, we observed a small but reproducible decrease in IgG1 production in the CFA protocol. Also, even though an early (48-h) increase in IL-4 mRNA expression was observed in Sm28-GST/Der p 1-immunized mice, no significant increase was observed at later time points at either the mRNA level or in terms of frequency of IL-4-producing cells. A possibility explaining this observation is that the Der p 1, in addition to affecting IFN- γ expression, also affects the expression of other cytokines that are involved in stimulating IL-4 expression. A good candidate for that is IL-2 since: 1) IL-2 has been shown to positively influence development of the IL-4-producing cells (34, 35), 2) IL-2 is a Th1-associated cytokine (36), and 3) IL-2 is central in the development of the immune response. According to this hypothesis, the small

diminution of the type 2 response at either the cytokine or the isotypic level is secondary to the substantial decrease of the Th1 function and the diminution of the IL-2 production. Our preliminary experiments support this hypothesis since Con A-stimulated spleen cells from Sm28-GST/Der p 1-immunized mice showed reduced capacity to produce IL-2, in comparison with mice immunized with Sm28-GST alone (data not shown). Another possibility is that the Der p 1 inhibits IL-12 production. IL-12, through its induction of IFN- γ production, can substantially increase synthesis and secretion of IgG2a. In addition, although large and sustained IL-12 responses inhibit IgG1 production (37), smaller and less sustained IL-12 responses can promote the secretion of all IgG isotypes, including IgG1 (38, 39). Thus, Der p 1 inhibition of a relatively limited IL-12 response would have precisely the effects that have been observed in the present study: marked inhibition of IgG2a production and modest inhibition of IgG1, with considerable inhibition of IFN- γ and a variable effect on IL-4 production. The proposed inhibitory effects of Der p 1 on IL-12 might result from Der p 1 induction of a cAMP-activating agent, such as PGE₂. In fact, other allergens (40) have been shown to elicit PGE₂, which through the induction of cAMP inhibit IL-12 production (41). Several other mechanisms may account for the observed effects of Der p 1 on cytokine expression. A recent observation, showing that Der p 1 induces changes in intracellular calcium flux on cultured human bronchial epithelial cells (C. King and G. Stewart, manuscript in preparation), brings up the possibility that Der p 1 affects cytokine production by inducing similar changes on T or other immunocompetent cells. Der p 1 may also be acting indirectly by modulating proinflammatory cytokine production by other cell types. In that respect, it is of interest that Der p 1 stimulates IL-6 production by epithelial cells (C. King and G. Stewart, unpublished observations), a cytokine that has been proposed to promote Th2 differentiation (42).

In addition of having similar effects in reducing IFN- γ expression in both the CFA and the alum protocol, Der p 1 had also distinct effects in these two protocols, especially on IgG1 production that was slightly reduced in the first and increased in the latter. A possible explanation for this is that Der p 1 differentially influence the cytokine pathways that are activated when mice are immunized using the CFA or the alum as adjuvants (43).

A possibility explaining the diminished anti-Sm28-GST response in mice immunized with Sm28-GST/Der p 1 in CFA is that the Sm28-GST is digested by the Der p 1, and as a consequence of that, diminished antigenic stimulation is provided. This possibility was examined in control experiments using the reference protease trypsin, which possesses a stronger protease activity than Der p 1. In the first place, we determined that mice coimmunized with Sm28-GST plus trypsin in alum produced sixfold higher anti-Sm28-GST IgG1 titers than mice immunized with Sm28-GST alone (data not shown). In addition, using the azocasein test, we have found that trypsin adsorbed in alum retained its full protease activity. Therefore, the presence of an active protease in the immunization mixture does not prevent the development of the immune response against an associated Ag. Finally, the migration pattern of Sm28-GST as demonstrated by PAGE did not change after incubation with Der p 1 (data not shown), suggesting that Der p 1 is unable to digest the Sm28-GST.

Previous studies have implied that the enzymatic activity of allergens is important for their antigenic properties. In fact, due to their enzymatic activity, allergens seem to cause epithelial damage in vitro (44). This finding implies that allergens may increase bronchial permeability and thereby enable Ag presentation to occur. It has also been shown that Der p 1 is able to cleave membrane-associated human CD23 to its soluble form (17, 18). Since soluble

CD23 positively influences IgE production (45, 46), it was proposed that through this mechanism, Der p 1 stimulates IgE responses. In a recent study, evidence was presented showing that the allergenicity of the bee venom phospholipase A₂ is dependent on its lipolytic activity, through which phospholipase A₂ induces mast cell degranulation and IL-4 secretion (40). In addition to the above findings, our study is the first to suggest that, due to its protease activity, an allergen, Der p 1, is able to modulate the immune response at an additional level, by influencing the balance between the antagonistic cytokines IL-4 and IFN- γ . However, since in most experiments Der p 1 was inactivated by heat treatment, which may affect the three-dimensional structure and the aggregation level of the Der p 1, we cannot exclude the possibility that the enzymatic activity of Der p 1 is not the only mechanism by which this protein affects cytokine expression. The use of recombinant Der p 1 lacking enzymatic activity may clarify this question.

It is noteworthy that Der p 1 was able to modulate the immune response against an irrelevant Ag in both immunization protocols. In the light of our results, it is likely that an initial allergic reaction may positively influence the development of type 2 responses against additional Ags. This may explain in part why many atopic individuals develop allergies to multiple substances. We are presently examining this issue with experiments that use SCID mice reconstituted with PBL from allergic individuals (47).

In conclusion, our data suggest that the major allergen, Der p 1, has the capacity 1) to induce type 2 responses by modifying the IL-4/IFN- γ balance in favor of the former and thereby to influence isotypic profiles, and 2) to influence a bystander immune response; also its protease activity is important in modulating the immune response.

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