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Alveolar macrophages and mast cells isolated from guinea pig lung were passively sensitized with IgG1, IgG2, or serum obtained from guinea pigs actively sensitized with ovalbumin. The release of histamine by mast cells and of thromboxane A2 by alveolar macrophages upon ovalbumin challenge indicated that both antibodies and serum were capable of sensitizing these cells with similar effectiveness. Heating the serum at 56°C for 4 h to inactivate IgE did not modify the antigen-dependent response of lung cells. These results suggest a predominant role for IgG in the allergic response of the guinea pig through the activation of different cell types such as lung mast cells and alveolar macrophages.

Key words: alveolar macrophages; IgG antibodies; mast cells.

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Mast cells are held responsible for type 1 hypersensitivity reactions after antigen inhalation. The IgE subclass of immunoglobulins (16) is said to account for the release of stored histamine after exposure to antigen. The mast cell is, however, unlikely to be the only effector cell, particularly since histamine antagonists reduce only to a very limited extent airway resistance in human asthma (14), and anaphylactic bronchoconstriction in the guinea pig can be made refractory to histamine and leukotrienes as well (12). In addition, IgG antibodies are involved in the release of leukotrienes (23) and histamine (9) from passively sensitized guinea pig lung parenchymal strips upon antigen challenge. *In vivo*, IgG antibodies are involved in the anaphylactic response observed in actively sensitized guinea pigs (5) and in airway obstruction mediated by LTD4 produced in response to PAF formation, following passive sensitization (34). When sensitized with ovalbumin, guinea pigs produce low levels of IgE and high levels of IgG antibodies (22). Higher amounts of IgG2 than IgG1 were observed in the serum from such animals (9). However, the amount of IgG and IgE produced can, to some extent, be modulated by the

sensitization protocol (2).

Guinea pig macrophages bear two distinct receptors for IgG, Fcγ₁/γ₂R, which binds both IgG1 and IgG2, and Fcγ₂R, which binds only IgG2 (26). Similar association constants have been described for both receptors, but the number of Fcγ₁/γ₂R molecules is two times higher than that of Fcγ₂R (25). Several FcγR have been also recognized on murine peritoneal mast cells and shown to trigger degranulation (4). Three classes of human Fcγ receptors have been described: FcγRI, which has high affinity for monomeric IgG, and two lower affinity receptors (FcγRII, FcγRIII) that interact with bound IgG or IgG in soluble immune complexes (18).

IgG antibodies have been shown to be mainly involved in phagocytosis (19) or antibody-mediated cytotoxicity (33) and in the recognition of foreign cells by macrophages (31).

Unlike those of IgE, IgG antibodies are not generally considered to participate in allergic reactions involving either alveolar macrophages or mast cells. Indeed, the prevalence in allergy of IgE, as compared with IgG, appears to be clear in both cell types from man and most animal species (29), although macrophages can be activated after cross-linking of IgG and IgE on their surface. Alveolar macrophages from guinea pigs actively sensitized with ovalbumin

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release large amounts of thromboxane A₂ (TxA₂) when challenged by the specific allergen, suggesting that these cells contribute to the allergic reaction by an antibody-mediated mechanism (3). Heating the serum (thus inactivating IgE but not IgG) from actively sensitized guinea pigs does not remove its capacity to sensitize passively guinea pigs *in vivo* or parenchymal lung strips *in vitro* (9). In addition, purified IgG1 and IgG2 can transfer passive sensitization to parenchymal lung strips as readily as does serum: smooth-muscle contraction accompanied by the release of histamine and by the formation of TxA₂ was observed upon challenge with ovalbumin (9). A cytophilic mechanism, analogous to the one which sensitizes mast cells and basophils for IgE-dependent release of histamine, can thus be suggested to cause cell activation in guinea pig lungs through the binding of a subclass of IgG immunoglobulins.

For exploration of this mechanism at the cellular level, guinea pig lung mast cells and alveolar macrophages were passively sensitized with guinea pig serum from actively sensitized animals or purified IgG1 or IgG2 antibodies directed against ovalbumin and separated by affinity chromatography from serum (6). The release of histamine from mast cells and of TxA₂ from purified alveolar macrophages was measured after challenge with the specific antigen (ovalbumin) or anti-IgG antibodies.

Material and methods

Animals, reagents, and sera

Hartley guinea pigs of both sexes, weighing 400–500 g, were used throughout the study. Collagenase (ref. C-9891) and bovine serum albumin (BSA, fraction V) were obtained from Sigma (USA). Heparin was kindly donated by Laboratoires Choay (France). Antiovalbumin IgG1 and IgG2 antibodies, purified by affinity chromatography from blood taken from actively sensitized guinea pigs, were kindly provided by Dr H. J. Showell (Pfizer Central Research, USA). Rabbit anti-guinea pig IgG was obtained from Biosys (France). Egg-white ovalbumin was obtained from ICN Immunological (USA). Radioimmunoassay kits for TxB₂ were purchased from Pasteur Diagnostics (France). Polyethyleneglycol 6000 was obtained from Merck (Germany). Orthophtaldehyde (OPTA) was obtained from Fluka (Switzerland). RPMI 1640 medium was purchased from Jacques Boy (France). Antibiotics (penicillin, streptomycin, and amphotericin B) were obtained from Gibco (USA). Reagents for the protein assay were obtained from Pierce (USA). IgG-rich antiserum for sensitization was produced by guinea pigs sensitized by subcutaneous injections on days 0 and 14 with ovalbumin (1 mg "sc") dissolved in 0.5 ml of 0.9% NaCl

containing 5 mg aluminum hydroxide. Blood was collected by cardiac puncture 1 week after the second injection, and serum was aliquoted and stored at –20°C.

Isolation of lung mast cells

Lung mast cells were isolated by collagenase digestion, as previously described (1). In brief, animals were killed by a sharp blow to the head and exsanguinated. The lungs were removed and dissected free of major airways and blood vessels, cut into small pieces with scissors, and further cut with a McIlwain tissue chopper. The chopped tissue (1 mm³) was then digested in prewarmed (37°C) BSA-Tyrode's buffer (0.1% BSA) containing collagenase (160 units/ml) for 90 min in a shaking water-bath. At the end of the digestion period, tissue fragments were disrupted by passage through a syringe (10 ml). The final suspension was then filtered through moistened gauze to remove tissue debris. The cells were recovered by centrifugation (70 g, 3 min, 4°C) and then washed once with BSA-Tyrode's buffer and resuspended in the required volume of the appropriate buffer. The cell viability, judged by the trypan blue exclusion test, was $60.5 \pm 1.5\%$ at the time of antigen challenge.

Passive sensitization and challenge of mast cells

Dispersed guinea pig lung mast cells (approximately 3×10^6 cells/ml) were suspended in BSA-Tyrode's buffer containing heparin (20 U/ml) and antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml, and amphotericin B 0.25 µg/ml). Purified IgG1, IgG2 (1–10 µg/ml), or serum (500 µl/ml), separated from blood of ovalbumin-sensitized animals, was added to the cell suspensions and incubated for 2 h at 37°C and then overnight at 4°C. The cells were washed twice with Tyrode's solution and resuspended in prewarmed Tyrode's buffer. Sensitized cells (900 µl) were added to the test secretagogue (100 µl), either ovalbumin (0.01–10 µg/ml, final concentration) or anti-IgG (0.01–10 µg/ml, final concentration). Secretion was allowed to proceed for 20 min at 37°C, and the reaction was terminated by adding ice-cold Tyrode's buffer (1 ml).

Histamine assay

The fluorescence obtained after condensation of extracted histamine with OPTA was measured as described by Lebel (17). Spontaneous release (approximately 4%) was always subtracted from all values before calculations of histamine release were made. The percentage of histamine release was calculated by the formula: $100(e-b)/(c-b)$, where e = mean of

the fluorometric readings for the experimental supernatant, b = mean of blanks, and c = mean of total histamine. For total histamine determination, an equivalent volume of the cell suspension was treated by perchloric acid.

Isolation of alveolar macrophages

Bronchoalveolar cells were collected from anesthetized guinea pigs (sodium pentobarbital, 40 mg/kg, i.p.) by 20 successive lavages with 5-ml aliquots of 0.9% NaCl. The lavages were centrifuged (475 g, 10 min at room temperature), and the cell pellet was resuspended in a culture medium (RPMI 1640), supplemented with HEPES buffer (10 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml). The cell suspensions were plated in 35-mm Petri dishes (2 ml per dish containing approximately $2-3 \times 10^6$ cells) and allowed to adhere for 1 h at 37°C in a humid atmosphere of 95% air and 5% CO₂. Nonadherent cells were discarded, and the remaining cells (>95% alveolar macrophages) were incubated for an additional hour in 1 ml RPMI medium.

Passive sensitization and challenge of alveolar macrophages

Adherent macrophages (>90% viable) were incubated with IgG1, IgG2 (5 µg/ml), or serum (500 µl/ml) in 1 ml of RPMI medium for 30 min at 37°C and then overnight at 4°C. At the end of the sensitization period, the supernatants were removed and, after washing, the monolayers were challenged in 1 ml RPMI with ovalbumin (0.01–100 µg/ml) or anti-IgG (0.01–100 µg/ml) for 10 min at 37°C. The supernatants were collected and stored at –20°C for TxA₂ assay.

Thromboxane A₂ assay

TxA₂ was measured as its stable derivative TxB₂ by radioimmunoassay according to the method of Sors et al. (28). Briefly, cell supernatants were diluted in phosphate buffer (10 mM, pH 7.4) and incubated (100 µl) overnight at 4°C with ¹²⁵I-labeled TxB₂ (100 µl) and anti-TxB₂ antiserum (100 µl) diluted in phosphate buffer containing bovine gamma globulin (0.3% w/v). Bound fractions were precipitated by a solution of polyethylenglycol 6000 (30%) and after centrifugation (2000 g, 10 min at 4°C), the radioactivity obtained in the pellet was counted. The monoclonal antibody employed was less than 0.02% cross-reactive with PGD₂, PGE₂, PGF_{2α}, 6-keto-PGF_{1α}, and arachidonic acid. The sensitivity of the assay was approximately 2 pg of immunoreactive TxB₂ in a 100-µl sample.

Additional methods

Cell viability was judged by the trypan blue (0.05% exclusion test. Mast cell counts were performed in cytocentrifuge preparations of the lung suspension (Hettich Universal, Germany). Smears were fixed for 30 min in Carnoy's fluid and stained for 5 h in 1% Alcian blue (11). The protein content in lung suspensions and in scraped macrophage monolayers was determined with bicinchoninic acid (27). The yield was approximately 5×10^6 cells/mg protein. IgG titers were measured in serum samples by enzyme-linked immunoassay (ELISA), as previously described (3). IgG titers were expressed as the sample dilution necessary to obtain 50% of the maximal absorption. IgE titers were tested by passive cutaneous anaphylaxis (PCA). Briefly, 0.1 ml of 1/10 to 1/2430 dilutions (in 0.9% NaCl) of the serum to be tested was injected subcutaneously into the flank of the guinea pig. Three days later, PCA reactions were induced by the intravenous administration of 1 ml of Evans blue dye (0.5%) containing 1 mg ovalbumin. No PCA reaction was observed with control serum. PCA reactions were also compared between IgG1 and IgG2 preparations injected in serial 10-fold dilutions (0.01 ng–100 µg).

Expression of the results

Data were analyzed by the one-tailed Mann-Whitney U test and are expressed as the mean ± SEM (standard error of the mean).

Results

Passive sensitization of lung mast cells with various concentrations of IgG1 and IgG2 antibodies

Lung suspensions containing $1.0 \pm 0.2\%$ mast cells, passively sensitized by antiovalbumin IgG1 or IgG2 (1–10 µg/ml), produced a bell-shaped curve of histamine release when challenged with ovalbumin (0.01–10 µg/ml) (Fig. 1). The maximum release amounted to 19% for IgG1 (5 µg/ml) (Fig. 1a) and to 17% for IgG2 (10 µg/ml) (Fig. 1b) of the total cellular content of the amine and was achieved in both cases with an ovalbumin concentration of 1 µg/ml. Cells were most effectively sensitized at a concentration of 5–10 µg/ml of antibody, and no significant differences were observed regarding the sensitizing ability of IgG1 or IgG2 antibodies. When tested for PCA reaction, a similar response was observed with both antibodies, and injection of 0.01–0.1 ng induced a positive reaction (1.5 cm for IgG1 and 1.3 cm for IgG2). Control cells, subjected to the same treatment as sensitized cells, were not affected by ovalbumin challenge (Fig. 1). Challenging the sensitized cells with anti-IgG also produced a bell-

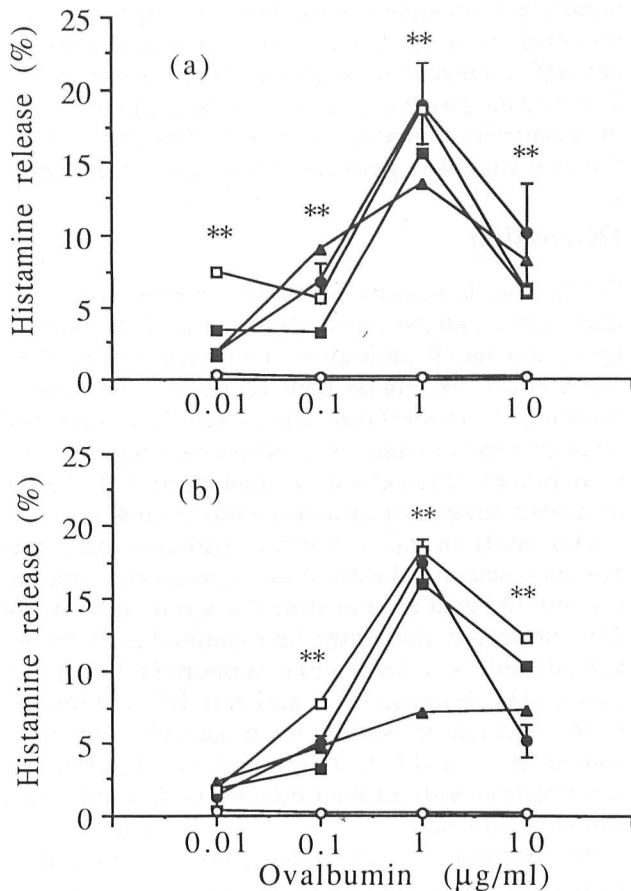


Fig. 1. Histamine released by lung mast cells passively sensitized by antiovalbumin IgG1 and IgG2 and challenged with ovalbumin. Release of histamine was measured in dispersed mast cells upon challenge with increasing amounts of ovalbumin (0.01–10 µg/ml, 20 min at 37°C). In (a), release of histamine was measured in control, nonsensitized cells (○) and in cells passively sensitized by 1 (■), 2 (▲), 5 (□), or 10 (●) µg/ml IgG1. In (b), release of histamine was measured in control, nonsensitized cells (○) and in cells passively sensitized by 1 (■), 2 (▲), 5 (□), or 10 (●) µg/ml IgG2. Results are the mean ± SEM from 4–7 experiments. ** $P < 0.01$, as compared with control, nonsensitized cells.

shaped curve, the maximum histamine release being achieved with 1 µg/ml anti-IgG (data not shown).

Passive sensitization of guinea pig lung mast cells with serum from ovalbumin-sensitized animals

The IgG titer of the serum pool obtained from sensitized guinea pigs was 1/80 000, as judged by an ELISA test, and no IgE antibodies were detected by a PCA test. Lung suspensions were sensitized with serum, as described above, and challenged with ovalbumin (Fig. 2a) or anti-IgG (Fig. 2b). In comparison with nonsensitized cells, serum-sensitized lung mast cells released higher amounts of histamine upon antigen challenge, which was unaffected by heating the serum (56°C, 4 h) before the sensitization procedure. Nonsensitized cells were unaffected by the

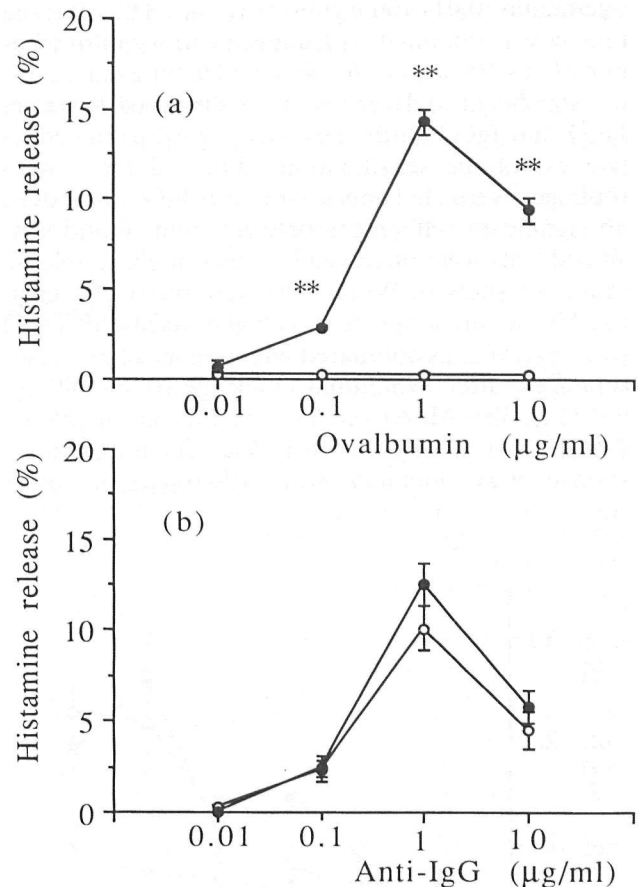


Fig. 2. Histamine released by lung mast cells passively sensitized by antiovalbumin IgG-rich serum and challenged with ovalbumin or anti-IgG. Release of histamine was measured in dispersed, control, nonsensitized mast cells (○) or cells sensitized with serum (●), upon challenge (20 min, 37°C) with increasing amounts of ovalbumin (0.01–10 µg/ml) (a) or anti-IgG (0.01–10 µg/ml) (b). Results are the mean ± SEM from 6–7 experiments. ** $P < 0.01$, as compared with control, nonsensitized cells.

ovalbumin challenge but responded to anti-IgG (Fig. 2b). Although the maximal histamine release triggered by anti-IgG in nonsensitized lung mast cells was lower than that observed for sensitized cells, the difference was below the level of statistical significance. The release of histamine was always dependent on extracellular calcium concentration: 90% inhibition of histamine release was observed when the challenge was performed in a standard buffer without added calcium (data not shown).

Passive sensitization of guinea pig alveolar macrophages with IgG1 and IgG2 antibodies or serum from sensitized animals

Alveolar macrophages passively sensitized by antiovalbumin IgG1 or IgG2 (5 µg/ml) antibodies released significantly higher amounts of TxA₂ than did control nonsensitized cells, when challenged with

ovalbumin (0.01–100 $\mu\text{g/ml}$) (Fig. 3a). The maximal release was obtained with concentrations of ovalbumin of 10–100 $\mu\text{g/ml}$. As observed in lung mast cells, no significant differences were observed between IgG1 and IgG2 antibodies with regard to the effectiveness of the sensitization. When alveolar macrophages were challenged with anti-IgG antibodies, no significant differences between control and sensitized cells were observed in terms of TxA2 release (data not shown). When cells were passively sensitized by serum, a significantly higher release of TxA2 was observed, as compared with nonsensitized macrophages, after ovalbumin challenge (0.01–100 $\mu\text{g/ml}$) (Fig. 3b). Moreover, heating the serum (56°C, 4 h) did not modify this response. The maximal response was obtained with 10–100 $\mu\text{g/ml}$ oval-

bumin, and no significantly different responses were obtained between control and sensitized cells for anti-IgG challenge (data not shown). The release of TxA2 from passively sensitized macrophages was only partially inhibited (approximately 30%) when the challenge was performed in a Ca^{2+} -free buffer.

Discussion

Guinea pig lung cells, including macrophages and mast cells, can be passively sensitized by purified IgG1 and IgG2 antibodies or by serum from allergic animals containing high IgG titers. Release of histamine from mast cells and of TxA2 from purified alveolar macrophages was observed when the previously sensitized cells were challenged with the appropriate antigen or anti-IgG immunoglobulins.

Our study revealed that the response from cells passively sensitized with serum from allergic animals was not reduced after heating the serum at 56°C for 4 h, indicating that only IgG antibodies were involved, unless a heat-stable subpopulation of IgE exists (24). Indeed, IgG, and not IgE, antibodies were detected in serum from actively sensitized guinea pigs by PCA determination. Accordingly, sensitization with purified IgG antibodies induced a similar response.

The optimal conditions for passive sensitization with serum or purified IgG antibodies were similar in both lung mast cells and macrophages. The sensitization was always more effective when performed over a long period (approximately 18 h) at 4°C (when phagocytosis was inhibited) (10). In agreement, the apparent association constant of $\text{Fc}\gamma\text{R}$ for IgG was shown to be considerably higher at 4°C than at 37°C in a murine macrophage-like cell line (32).

The observed release of histamine and TxA2 from pulmonary cells appeared to be dependent on the binding of antibodies to the receptors, and both processes seem to be relevant to a similar IgG-dependent mechanism. Although murine peritoneal mast cells bear receptors of IgG (4), no direct studies are known on the binding and function of IgG in lung mast cells, and histamine release may be helped in lung suspensions by other cells that produce activating factors for mast cells. In dispersed guinea pig lung mast cells, the release of histamine by various secretagogues has been shown to be unaffected by the degree of mast cell purification (30), suggesting a negligible influence of other cell types on *in vitro* histamine release. However, caution in reaching this conclusion must be expressed, considering the low degree of mast cell purification (1%) in the lung suspensions. The biphasic nature of the curves may result from a gradual increase in the number of cross-links between antigen or anti-IgG and

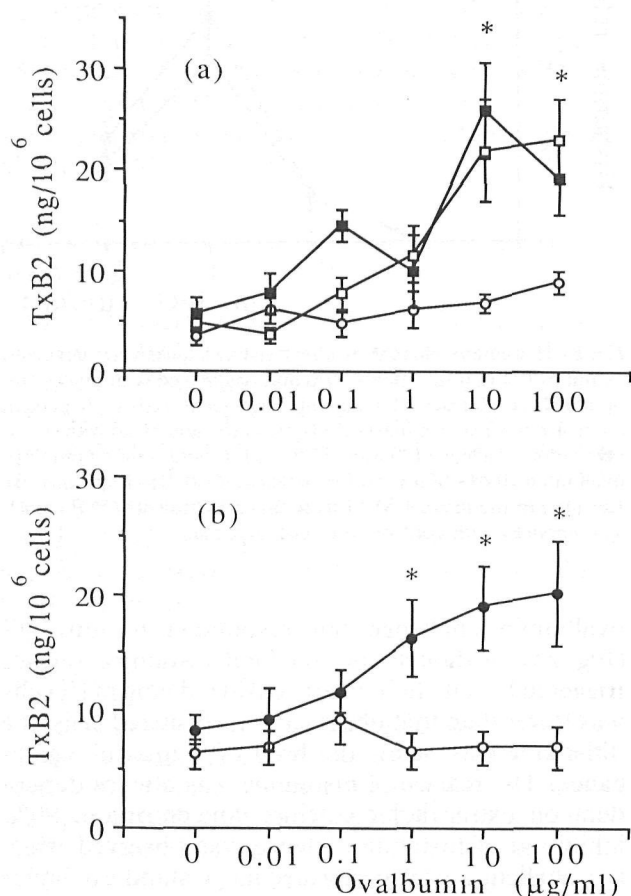


Fig. 3. Thromboxane A2 released by alveolar macrophages passively sensitized by antiovalbumin IgG1, IgG2, or IgG-rich serum and challenged with ovalbumin. Release of TxA2 was measured from adherent alveolar macrophages upon challenge with increasing amounts of ovalbumin (0.01–100 $\mu\text{g/ml}$, 10 min 37°C). In (a), TxA2 was measured in control, nonsensitized (○) or macrophages sensitized by IgG1 (■) or IgG2 (□) (5 $\mu\text{g/ml}$). In (b), TxA2 was measured in control, nonsensitized (○) or macrophages sensitized with serum (●) (500 $\mu\text{l/ml}$). Results are the mean $\pm$ SEM from six experiments. * $P < 0.05$, as compared with control, nonsensitized cells.

membrane-bound IgG and the inhibition of cross-linking by excess of antigen, leading to the decrease in histamine release (8) as described for histamine release curves with bivalent penicilloyl haptens.

Our experiments did not demonstrate significant differences in the effectiveness of the two IgG antibodies, since similar histamine or TxA₂ release was obtained from cells passively sensitized under the same conditions with either IgG1 or IgG2 antibodies. However, differences in the roles and in the effectiveness of both IgG receptors have been described and remain controversial. Early studies on passive cutaneous anaphylaxis reported that IgG1, but not IgG2, antibody may passively sensitize guinea pig skin, probably because cutaneous mast cells lack Fc γ ₂ receptors (21). Later on, however, a number of monoclonal mouse anti-IgG2 antibodies were also reported to degranulate peritoneal mast cells (7, 13). The most likely explanation for this discrepancy lies in the fact that mast cells from different locations exhibit marked variations in their functional properties as well as in the Fc γ R they express (3). In agreement with our observations, phagocytosis of ovalbumin complexes of IgG by guinea pig peritoneal macrophages has been found to proceed more efficiently through Fc γ ₁/ γ ₂R (which binds IgG1 and IgG2 with similar affinity) than Fc γ ₂R (which binds only IgG2) (26). In contrast, Fc γ ₂R triggers calcium mobilization more intensively than Fc γ ₁/ γ ₂R in the same cells (15), and the activation of phospholipase A₂ is more efficiently transmitted by the binding of IgG2b to its specific receptor in a murine macrophage cell line (20). Besides, in parenchymal lung strips from guinea pigs, sensitization with IgG1 induced a markedly higher smooth-muscle contraction than with IgG2 (9). Contamination of the different IgG fractions or variability in the signals transmitted by the binding of either antibody between individual cell types may explain these different observations.

The response to an antigen challenge was always highly specific in this study, in striking contrast to the response to anti-IgG, since in purified alveolar macrophages the response to an anti-IgG challenge was negligibly increased by sensitization with either serum or IgG, and in lung mast cells, sensitization increased the response only slightly. The presence of *in vivo* bound IgG antibodies, particularly in alveolar macrophages from normal guinea pigs, is probably the explanation for this observation, reflecting the finding that IgG antibodies bind to guinea pig macrophages with high affinity (the association constant of Fc γ ₁/Fc γ ₂R for ovalbumin-specific IgG2 is $1.4 \times 10^{-8} \text{ M}^{-1}$) (19).

By the use of isolated cell systems, the present study strengthens the evidence for a role of IgG1 and IgG2 antibodies in the allergic response of the guinea

pig and demonstrates the involvement of alveolar macrophages in this process.

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