

Induction of IL-4-dependent, anaphylactic-type and IL-4-Independent, non-anaphylactic-type IgG1 antibodies is modulated by adjuvants

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Abstract

Adjuvants can modulate the levels of anaphylactic- and non-anaphylactic-type IgG1 antibodies produced in response to a particular antigen. Mice immunized with ovalbumin (OVA) in Al(OH)₃ gel (alum) produced mostly the anaphylactic type, irrespective of the s.c. or i.p. route used, and this antibody was not detectable in IL-4^{-/-} mice. In contrast, when OVA was injected in complete Freund's adjuvant (CFA), it induced substantial amounts of non-anaphylactic-type IgG1 in both IL-4^{+/+} and IL-4^{-/-} mice, and some anaphylactic IgG1 antibody in IL-4^{+/+} mice only. When IFN- γ was neutralized by specific mAb in wild-type mice immunized with OVA in CFA, the anaphylactic-type IgG1 antibody increased reaching the same levels as in alum-injected mice. This result indicates that the induction of IFN- γ by the immunization with CFA down-regulates the production of IL-4-dependent, anaphylactic-type IgG1. Despite their different effects on IgG1 antibody production, both adjuvants dramatically increased the production of IgG2a in IL-4-deprived mice and did not induce any detectable IgE in these mice.

Introduction

Adjuvants have the ability to enhance and also to selectively drive the immune response to different antigens. These properties have been considered in many vaccination strategies to evoke an effective cellular or humoral immune response to pathogens (1–3). Studies focused on the induction of a humoral immune response to soluble antigens demonstrated that differences in the pattern of antibody isotypes produced depend upon the biochemical characteristics of the adjuvants (4,5). More specifically, Al(OH)₃ gel (alum) and incomplete Freund's adjuvant (IFA) promote IgE and IgG1 antibody production, whereas some types of liposomes induce antigen-specific IgG2a, IgG2b and IgG3 antibodies. On the other hand, complete Freund's adjuvant (CFA) and saponin promote the synthesis of IgG1 and IgG2a in response to T-dependent antigens (6–10). Different cytokines secreted by T_H1 and T_H2 cells play a crucial role in determining both the nature and strength of antibody responses (11–15). Therefore, the immunomodulatory properties of adjuvants can depend on their ability to up-regulate T_H1- or T_H2-type immune responses (1,3,8,10).

We have recently shown that two distinct components obtained by fractionation of a worm extract induce the production of two types of IgG1 antibodies when mice are immunized with these antigens in association with CFA. These antibodies differ in their ability to elicit mast cell degranulation, as measured by passive cutaneous anaphylaxis (PCA), and their synthesis is regulated by different cytokines. The population of IgG1 antibodies which displays anaphylactic activity is dependent of IL-4, like IgE antibodies, whereas non-anaphylactic-type IgG1 is stimulated by IL-12 and/or IFN- γ . These two cytokines are also responsible for the down-regulation of the anaphylactic-type IgG1 (16).

The complexity of the biological functions of IgG antibodies which are related to their Fc fragments, such as anaphylaxis, cell lysis and opsonization, was first described in the guinea pig in 1963 (17–19). This was further extended to man and other animal species, and heterogeneity even among antibodies of the same subclass has been reported. For example, mouse IgG1 antibodies were divided in two sub-

populations according to the ability to activate the complement system or to their PCA activity in mice and rats (20,21).

In this work we demonstrate that synthesis of the two types of mouse IgG1 antibodies, previously described by us (16), in response to one kind of antigen can be modulated by adjuvants. Alum induced mostly the production of IL-4-dependent, anaphylactic-type IgG1 antibodies, whereas CFA promoted the synthesis of both types of antibodies. We also show that the levels of anaphylactic-type IgG1 in CFA-injected mice were impaired by the induction of IFN- γ .

Methods

Animals

BALB/c mice that had targeted disruption of the IL-4 gene (IL-4^{-/-}), provided by Dr L. V. Rizzo (Institute of Biomedical Sciences, University of São Paulo), and wild-type BALB/c (IL-4^{+/+}) mice were bred and maintained at the animal house facilities of the Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, under specific pathogen-free conditions. Groups of five to six female age-matched mice were used for immunization protocols. BALB/c mice (5–6 weeks old) and Wistar rats (3 months old) were used for PCA reactions.

Immunization protocols

Ovalbumin (OVA) grade V from Sigma (St Louis, MO) in association with CFA (Sigma) or alum (Fontoura Wyeth, São Paulo, Brazil) was used in the immunization protocols.

Two groups of six animals were immunized with OVA (150 μ g/animal) + CFA s.c., in the base of the tail, or in association with alum at 7.5 mg/animal, i.p. These groups were killed by CO₂ inhalation and bled from the heart 8 or 10 days after immunization respectively.

In another experiment, a similar protocol was used to immunize two groups of five IL-4^{+/+} or IL-4^{-/-} mice. In addition, two other groups of five IL-4^{+/+} or IL-4^{-/-} mice were injected s.c. with the same amounts of OVA (150 μ g/animal) and alum (7.5 mg/animal), and bled after 8 days.

In vivo IL-4 or IFN- γ neutralization

For *in vivo* IL-4 neutralization, groups of five BALB/c mice were immunized with OVA + alum, i.p., and treated with three i.p. injections (3 mg each/animal) of anti-IL-4 mAb 11B11 or isotype control mAb GL 113 [anti- β -galactosidase (β -gal)] on days 0, 3 and 6 of immunization.

For IFN- γ neutralization, other groups of mice immunized with OVA + CFA were treated with two injections (1 mg each/animal) of anti-IFN- γ mAb XMG 1.2 or isotype control mAb GL 113 (anti- β -gal) on days -1 and 3 of immunization.

Titration of antibodies by ELISA

The levels of IgG1 and IgG2a in plasma from previously immunized mice were determined by ELISA using plates coated with OVA and biotinylated goat anti-mouse IgG1 or IgG2a antiserum (Southern Biotechnology Associates, Birmingham, AL). The reactions were developed with streptavidin-peroxidase conjugate (ExtraAvidin; Sigma), o-phenylenediamine and H₂O₂. The plates were read at 450 nm on an

automated ELISA reader (Dynatech, Chantilly, VA). The anti-OVA IgE antibodies were measured by capture-ELISA using plates coated with rat anti-mouse IgE and biotinylated OVA. The reactions were developed as described for IgG1 and IgG2a.

Titration curves were conducted for all samples and the results are shown as the mean OD of five or six samples per group ($\pm$ SEM) at various plasma dilutions. ANOVA followed by multiple comparisons using the Tukey method (22) was employed to compare the different groups. Some samples were quantified by comparison with a standard curve of a monoclonal mouse IgG1 antibody.

PCA

The anaphylactic activity of IgG1 was evaluated by PCA reactions in mice as described by Ovary (23). The mice were previously shaved and injected intradermally (50 μ l) with three serial dilutions of plasma (inactivated for 1 h at 56°C) in each side of the dorsal skin. After 2 h they were challenged i.v. with 250 μ g of OVA + 0.25% of Evans blue solution. For the IgE titration, PCA reactions were performed in rats using non-inactivated plasma, according to Mota and Wong (24).

All tests were made in triplicate and the PCA titers were expressed as the reciprocal of the highest dilution that gave a lesion of >5 mm in diameter. The detection threshold of the technique was established at 1:5 dilution.

Results

Preferential induction of IL-4-dependent IgG1 and IgE antibodies by alum in OVA-immunized mice

CFA and alum are known to stimulate T_H1 and T_H2 responses respectively (25). While CFA is often injected s.c., we observed in previous experiments that the isotype profile of antibodies produced when alum is injected s.c. is the same as that obtained when it is given i.p., but the antibody levels are much lower. Therefore, we chose the s.c. and i.p. routes to immunize the animals with CFA and alum respectively. The pattern of OVA-specific antibodies (Fig. 1A–C) shows that alum stimulated high levels of IgG1 and IgE and CFA of IgG2a, but also some IgG1, as measured by ELISA. In addition, the results of PCA reactions developed in mice and rats to test the ability of IgG1 and IgE to respectively degranulate their mast cells indicated that antibodies with anaphylactic activity were abundant in plasma of alum-injected mice (Fig. 2A and B). Since IgG1 is partially dependent upon IL-4 and can be also made in the absence of this cytokine (15,16,26–28), we treated mice immunized with OVA and alum with anti-IL-4 mAb in order to analyze if their elevated IgG1 antibody response was IL-4-dependent or -independent. Indeed, as we can see in Fig. 3, the synthesis of almost all OVA-specific IgG1 antibodies was abolished in IL-4-depleted mice, indicating that most of the IgG1 switching in alum-injected mice is induced by IL-4.

Differential stimulation of anaphylactic and non-anaphylactic type IgG1 by CFA and alum

As previously shown, the synthesis of anaphylactic- and non-anaphylactic-type IgG1 depends primarily upon the nature of

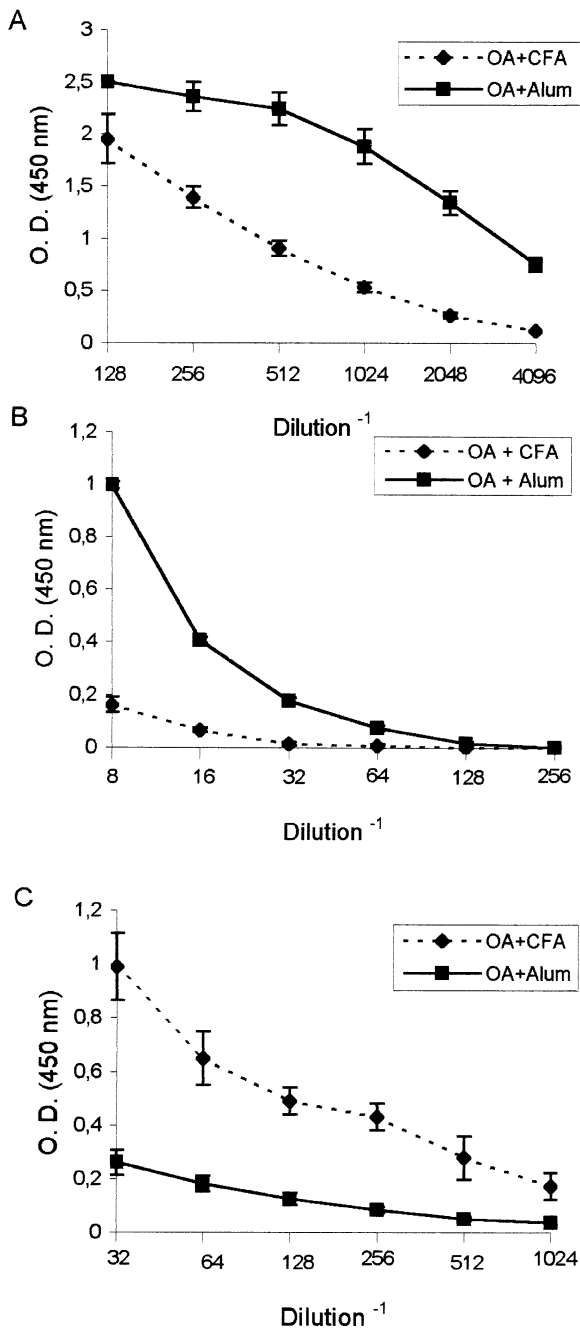


Fig. 1. OVA-specific IgG1 (A), IgE (B) and IgG2a (C) antibody production in BALB/c mice immunized with OVA in alum (i.p.) or CFA (s.c.) and bled after 10 or 8 days respectively. The results represent the mean OD of plasma dilutions from six mice per group ($\pm$ SEM).

the antigen (16). However, other factors, such as adjuvants, could change the pattern of cytokines, and consequently of antibody isotypes, synthesized in the immune response to an antigen. Therefore, we decided to compare the proportion of these two types of antibodies that are induced by the same antigen when injected with different adjuvants. Since the anaphylactic type is IL-4 dependent and the non-anaphylactic

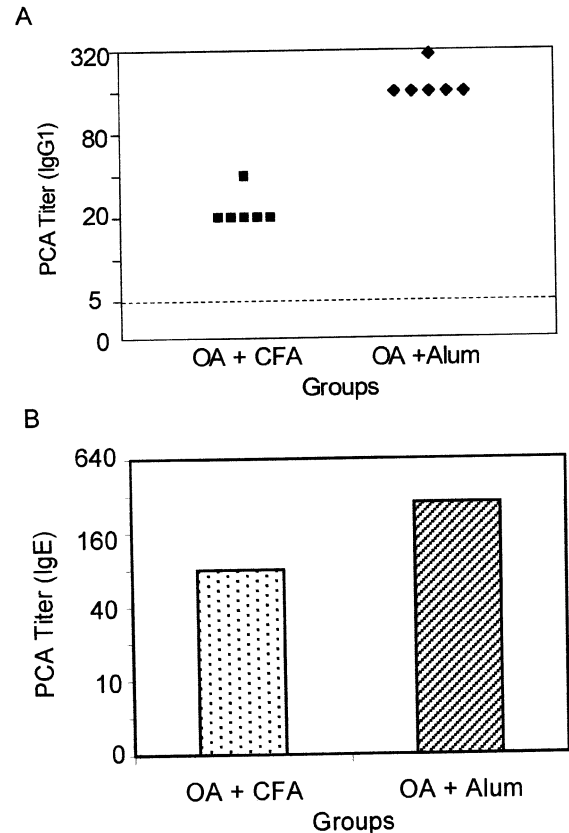


Fig. 2. Anaphylactic IgG1 (A) or IgE (B) antibody produced by BALB/c mice immunized with OVA in alum (i.p.) or CFA (s.c.) and bled after 10 or 8 days respectively. PCA titers represent the reciprocal of the highest dilution of heat-inactivated individual plasma for IgG1 or pooled plasma for IgE, which gave a lesion of >5 mm in diameter. The dashed line represents the detection threshold.

IL-4 independent, we immunized IL-4^{+/+} and IL-4^{-/-} mice with OVA in CFA or alum. A group injected with alum s.c. was also included to check if the route could have any influence on the proportion of anaphylactic and non-anaphylactic IgG1 antibodies produced. Figures 4(A), 5(A) and 6(A) show that substantial amounts of IL-4-independent IgG1 were produced by IL-4^{-/-} mice injected with CFA, but they were negligible in those injected with alum. In fact, plasma from the former group contained 14.25 ± 1.6 μ g/ml of OVA-specific IgG1, whereas those from the latter contained 2.34 ± 0.4 , when alum was given i.p., and 0.06 ± 0.0 μ g/ml, when it was given s.c. Plasma from the respective wild-type groups of mice contained 21.71 ± 0.35 (CFA), 24.85 ± 2.0 (alum i.p.) and 4.68 ± 0.2 (alum s.c.) μ g/ml. These results indicate, therefore, that CFA induced higher amounts of non-anaphylactic-type IgG1 than alum, irrespective of the route used. In contrast, Fig. 7(A–C) shows that alum induced more of the anaphylactic type when given i.p. compared with alum s.c. or CFA. As expected, this type of antibody was not detected in the absence of IL-4 in any of the three groups. The IgE antibody production followed the same pattern of anaphylactic IgG1. No IgE was obtained in any of the IL-4^{-/-} mice, irrespective of the adjuvant used (data not shown). Regarding IgG2a

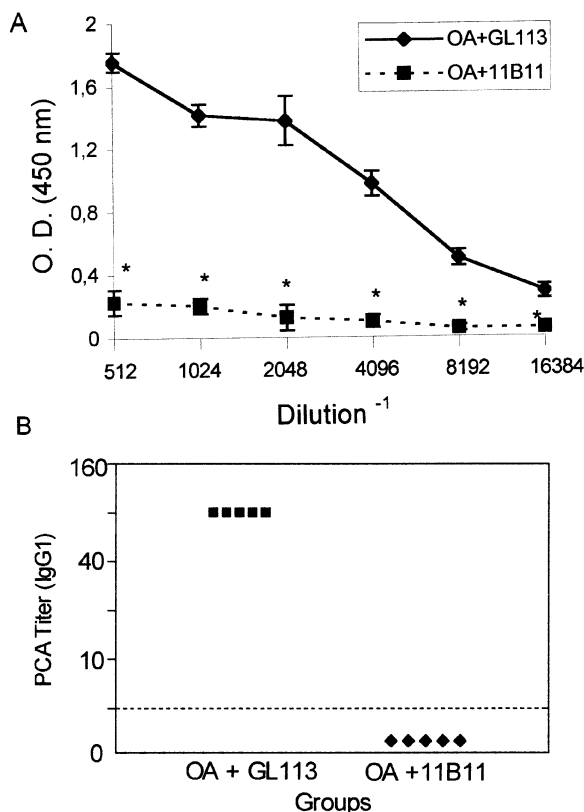


Fig. 3. OVA-specific IgG1 measured by ELISA (A) or PCA (B) in heat-inactivated plasma from BALB/c mice immunized with OVA in alum i.p. and treated with anti-IL-4 mAb (OVA + 11B11) or isotype control mAb (OVA + GL113). * $P < 0.05$ compared with isotype control group.

antibody production, inoculation of IL-4^{-/-} mice with antigen prepared with either CFA or alum resulted in dramatically increased levels of this isotype (Figs 4B, 5B and 6B).

Negative control of anaphylactic-type IgG1 by IFN- γ in mice immunized with OVA + CFA

Since CFA stimulates the production of higher levels of IFN- γ than alum in OVA-immunized mice (29) and this cytokine directly antagonizes the effects of IL-4 on B cells (30), we next investigated the role of IFN- γ in the final outcome of anaphylactic IgG1 levels in CFA-injected mice. Two groups of mice were treated with anti-IFN- γ mAb XMG 1.2 or isotype control anti- β -gal mAb GL 113 and immunized with OVA in CFA. Figure 8(A) shows that the IFN- γ -depleted mice produced 4 times more anaphylactic-type IgG1 than the control group of mice treated with GL 113 mAb. Furthermore, these antibody levels were comparable to those obtained in mice treated with the isotype control mAb and immunized i.p. with alum (Fig. 3B). When IgG1 was measured by ELISA (Fig. 8B), there was no difference between IFN- γ -depleted and -undepleted groups. The low levels of IgG2a antibodies in the former group, however, confirmed that IFN- γ was efficiently neutralized by treatment with mAb XMG 1.2 (Fig. 8C).

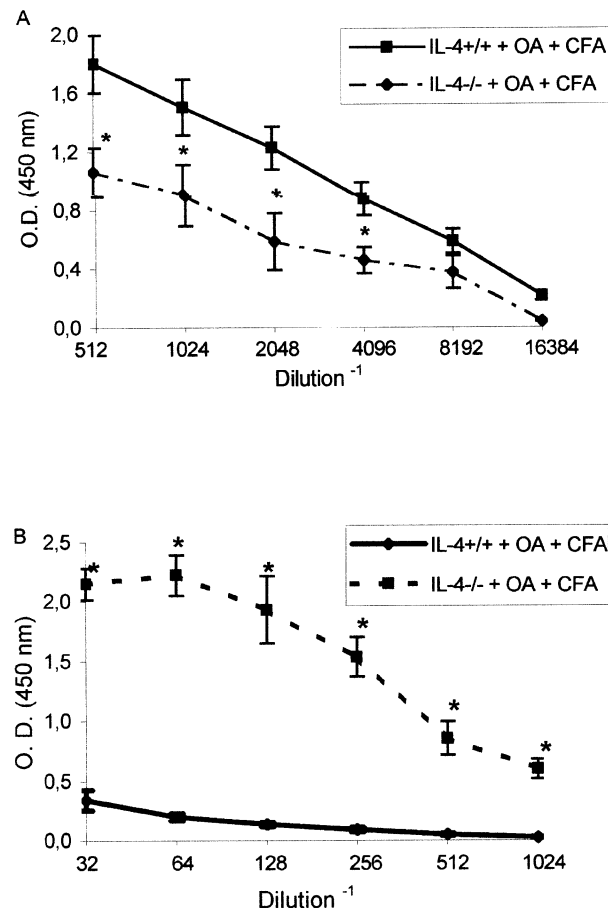


Fig. 4. OVA-specific IgG1 (A) and IgG2a (B) antibody production in IL-4 gene-disrupted (IL-4^{-/-}) or wild-type (IL-4^{+/+}) mice immunized with OVA in CFA s.c. and bled 8 days later. The results represent the mean OD of plasma dilutions from five mice per group ($\pm$ SEM). * $P < 0.05$ compared with IL-4^{+/+} mice.

Discussion

The ability to mount an IgG1 response is usually associated with the induction of a T_H2 response (31–33), although the synthesis of IL-4-independent IgG1 antibodies has been reported under several experimental conditions (26–28). Brewer and collaborators (29) showed that in IL-4-deficient mice injected with antigen prepared in CFA the production of IgG1 antibodies, although reduced, was nonetheless significant. Since CFA was unable to induce IL-5 in these mice, they presumed that it stimulated IgG1 production in a T_H2-independent manner. In this sense, we have recently demonstrated (16) that the synthesis of IL-4-independent IgG1 antibodies is stimulated by IL-12 and/or IFN- γ , and these molecules lack any anaphylactic activity. In contrast, the IL-4-dependent IgG1 antibodies which are induced by T_H2 cells have the ability to elicit mast cell degranulation.

Adjuvants are known to drive the immune response to T_H1 or T_H2 and while CFA is capable of stimulating classical cell-mediated immune responses, associated with expansion of T_H1 cells, alum is a potent T_H2 stimulator. In the present study,

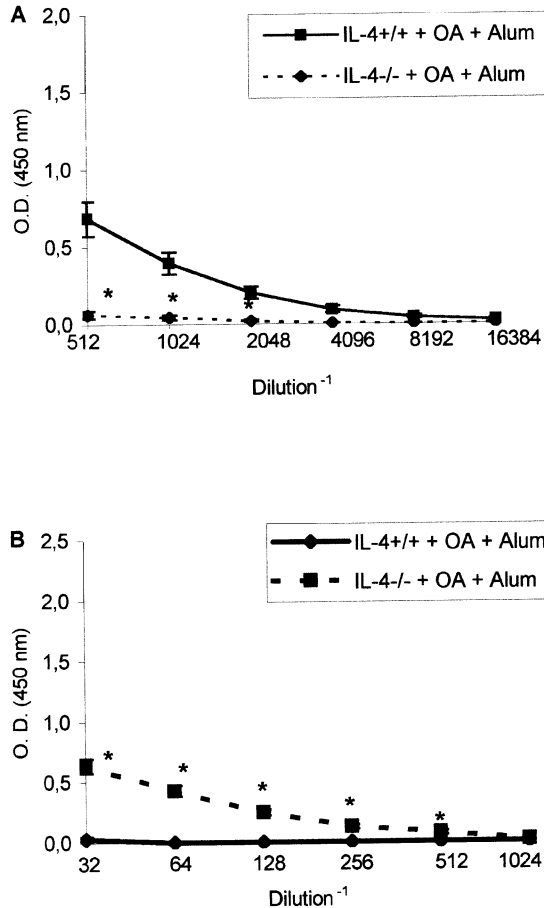


Fig. 5. OVA-specific IgG1 (A) and IgG2a (B) antibody production in IL-4 gene-disrupted (IL-4^{-/-}) or wild-type (IL-4^{+/+}) mice immunized with OVA in alum s.c. and bled 8 days later. The results represent the mean OD of plasma dilutions from five mice per group ($\pm$ SEM). * $P < 0.05$ compared with IL-4^{+/+} mice.

we found that these adjuvants can modulate the proportion of the two types of IgG1, mentioned above, induced by immunization with the same antigen. Thus, our results show that mice injected with alum produced mostly the anaphylactic type, which could not be detected in plasma of IL-4-deprived mice. In contrast, CFA induced IL-4-dependent and substantial amounts of IL-4-independent IgG1.

The former results are different from those reported by Brewer *et al.* (29) who obtained lower, but significant titers of IgG1 in IL-4^{-/-} mice inoculated with alum-adsorbed OVA. This difference could not be due to the route of immunization, since we also injected IL-4^{-/-} mice s.c. with alum and they did not produce any IgG1. However, the fact that we were looking at a primary response and they used an immunization protocol with a boosting inoculation on day 14 could probably explain the discrepancy in the results, since IL-4 is much less required to generate a secondary antigen-specific IgG1 response (34). Regarding IgG2a antibody production, our results, like those previously reported by others (29,35), demonstrate that, in the absence of IL-4, this isotype is greatly enhanced by immunization with either alum or CFA. This

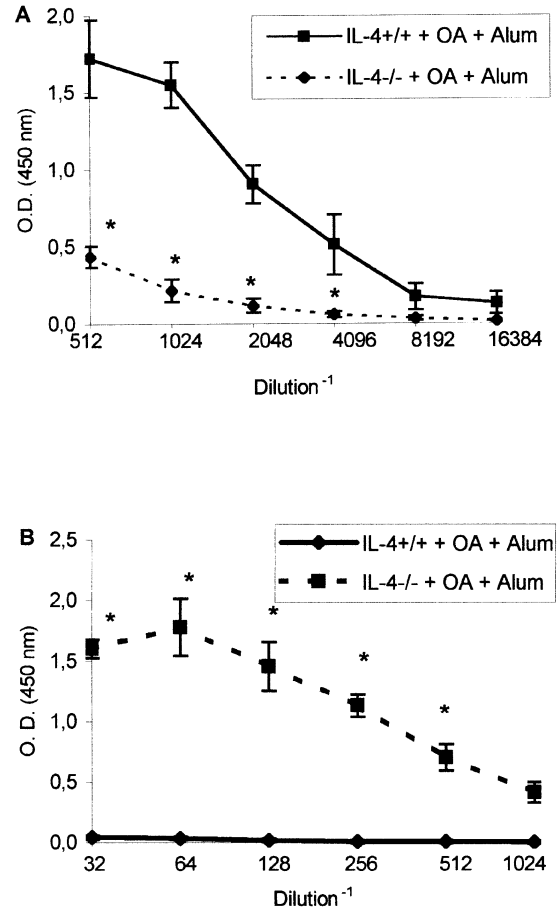


Fig. 6. OVA-specific IgG1 (A) and IgG2a (B) antibody production in IL-4 gene-disrupted (IL-4^{-/-}) or wild-type (IL-4^{+/+}) mice immunized with OVA in alum i.p. and bled 10 days later. The results represent the mean OD of plasma dilutions from five mice per group ($\pm$ SEM). * $P < 0.05$ compared with IL-4^{+/+} mice.

effect arises due to the ability of IL-4 to inhibit IFN- γ production and to antagonize its effect on B cells. Reciprocally, IFN- γ inhibits IL-4-mediated IgG1 and IgE isotype switching (36). In fact, when we neutralized IFN- γ in mice injected with CFA using anti-IFN- γ mAb, we observed an increase in anaphylactic-type IgG1, which is under IL-4 control, and a decrease in IgG2a, which is regulated by IFN- γ . It is interesting also to notice that IFN- γ -depleted mice injected with CFA can produce as much anaphylactic-type IgG1 as IFN- γ -undepleted, alum-injected mice (i.p.). These results indicate that the production of this type of antibody is under the negative control of IFN- γ stimulated in greater amounts by CFA under normal immunization conditions. The increased levels of anaphylactic IgG1 obtained in the absence of IFN- γ , however, were not apparently detected by ELISA, since the titration curves were similar in plasma from IFN- γ -depleted and -undepleted mice. These results could be explained by a decrease in the amounts of the non-anaphylactic-type IgG1, which as we showed before (16) is positively regulated by IFN- γ . This reduction would compensate for the increase in the anaphylactic-type IgG1. Indeed, the absence of IFN- γ or

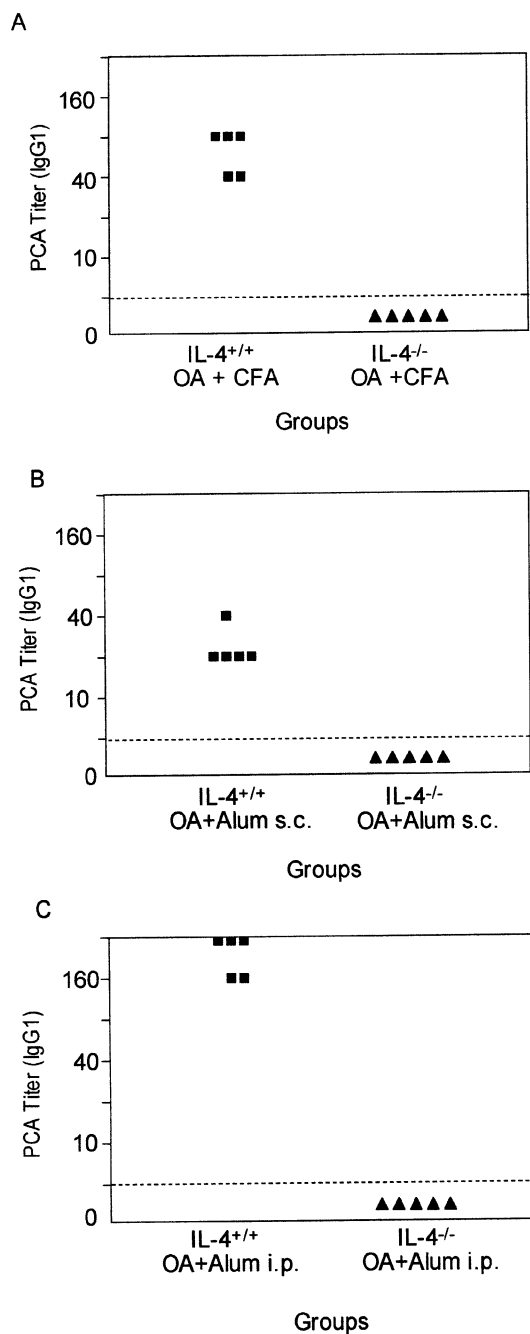


Fig. 7. Anaphylactic IgG1 antibody produced by IL-4 gene-disrupted (IL-4^{-/-}) or wild-type (IL-4^{+/+}) mice immunized with OVA in CFA (A) or alum s.c. (B) or i.p. (C). PCA titers represent the reciprocal of the highest dilution of heat-inactivated plasma which gave a lesion of >5 mm in diameter. The dashed line represents the detection threshold.

IL-12 leads to a decrease in IgG1 levels effectively detected by the ELISA procedure, when antigens that induce mostly the non-anaphylactic-type IgG1, like high mol. wt (PI) components from *Ascaris suum*, are used (16). This result is obtained despite the substantial increase in PI-specific, anaphylactic IgG1 antibodies that also occurs under such conditions.

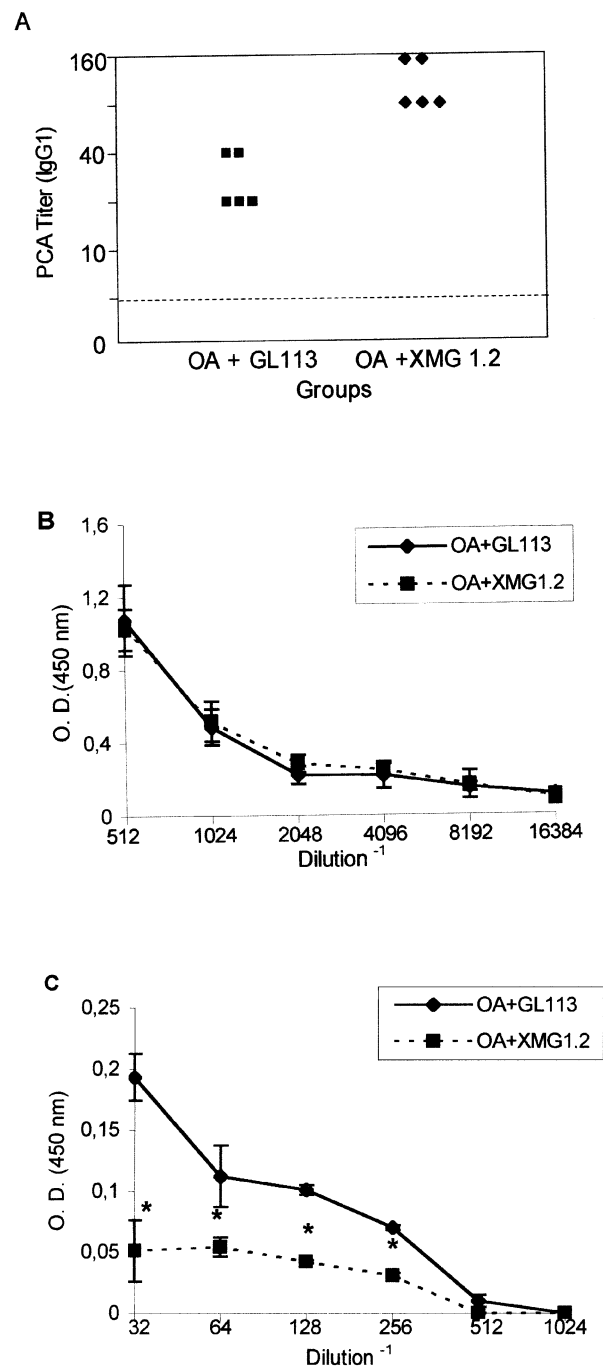


Fig. 8. OVA-specific IgG1 (A and B) and IgG2a (C) antibody produced by BALB/c mice immunized with OVA in CFA and treated with anti-IFN- γ (OVA + XMG1.2) or isotype control (OVA + GL113) mAb. PCA titers represent the reciprocal of the highest dilution of heat-inactivated plasma which gave a lesion of >5 mm in diameter. The dashed line represents the detection threshold. The ELISA results represent the mean OD of plasma dilutions from five mice per group ($\pm$ SEM). * $P < 0.05$ compared with isotype control group.

An intriguing aspect of the isotype response in alum- and CFA-injected mice is the inverse relationship between the induction of IgG2a and anaphylactic-type IgG1. Since the

former isotype can block IgG1-mediated PCA reactions in mice (37,38), we have used IgG2a/IgG2b-depleted plasma and demonstrated that the titers of anaphylactic IgG1 antibody did not change in these plasma (16). In addition, we observed in the present study that plasma from IL-4-deprived mice injected with OVA in alum did not have IgG1 antibody, detectable by ELISA, in contrast to substantial amounts present in those from CFA-injected mice. These findings altogether exclude the possibility that absence of IgG1-mediated PCA reactions, when plasma from IL-4^{-/-}, CFA or alum-injected mice are used, is due to an inhibitory activity of IgG2a, present in similar amounts in these same plasma.

Although it is clear that the different antibody-stimulating properties of alum and CFA reside in their ability to preferentially induce IL-4 and IFN- γ , the structural difference between anaphylactic and non-anaphylactic IgG1 antibodies induced by these same cytokines has not yet been identified. However, there is some evidence that the biological activity of these two types of IgG1 depends upon N-glycosylation of their Fc regions. In this sense, we have observed that when a mouse hybridoma cell line is cultivated in the presence of tunicamycin (N-glycosylation inhibitor), it secretes aglycosylated, DNP-specific IgG1 mAb that lack the ability to elicit an anaphylactic reaction compared with those produced by the same hybridoma cells in the absence of tunicamycin. In addition, IgG1 mAb with or without anaphylactic activity differ in their binding capacity to lectins, indicating that they have qualitative and/or quantitative differences in N-linked oligosaccharides (E. L. Faquim-Mauro *et al.*, manuscript in preparation).

In conclusion, our data demonstrate that the synthesis of IL-4-dependent, anaphylactic-type or IL-4-independent, non-anaphylactic-type IgG1 in response to an antigen can be determined not only by its nature, but also by the adjuvant activity of the carrier. Moreover, the ability of adjuvants to selectively induce one or another type of IgG1 is highly dependent on the balance in the production of IL-4 and IFN- γ .

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Abbreviations

alum	aluminium hydroxide gel
β -gal	β -galactosidase
CFA	complete Freund's adjuvant
IFA	incomplete Freund's adjuvant
OVA	ovalbumin
PCA	passive cutaneous anaphylaxis

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