Brief Definitive Report

Chemically Modified Antigen Preferentially Elicits Induction of Th1-like Cytokine Synthesis Patterns In Vivo

By Xi Yang, Randall S. Gieni, Tim R. Mosmann,[‡] and Kent T. HayGlass

From the Department of Immunology, University of Manitoba, Winnipeg R3E 0W3; and the [‡]Department of Immunology, University of Alberta, Edmonton T6G 2H7, Canada

Summary

Differential activation of CD4$^+$ T cell subsets in vivo leads to the development of qualitatively different effector responses. We identify an approach that allows selective activation of strongly Th1-dominated immune responses to protein antigens. Whereas in vivo administration of ovalbumin (OVA) induces cytokine synthesis that is neither Th1 nor Th2 dominated, administration of glutaraldehyde polymerized, high relative molecular weight OVA (OA-POL) leads to 20-fold increase in the ratio of interferon γ (IFN-γ)/IL-4 and IFN-γ/IL-10 synthesis observed after short-term, antigen-mediated restimulation directly ex vivo. In contrast, concurrent in vivo administration of anti-IFN-γ mAb and OVA or OA-POL results in marked increases in IL-4 and IL-10, and decreased IFN-γ production, reflecting a polarization of the response towards a Th2-like pattern of cytokine synthesis. These observations may be useful in clinical settings including hypersensitivity, autoimmune diseases, and vaccine development where the ability to actively select specific patterns of cytokine gene expression would be advantageous.

The pattern of cytokine production elicited after in vivo exposure to antigen largely determines the type of effector immune responses that develop (1–4). Many antigen-driven responses are characterized by a reciprocal relationship between delayed hypersensitivity and antibody production (5, 6). Helper T cell clones can be classified as Th0, Th1, and Th2 on the basis of function and the pattern of cytokine gene expression exhibited after polyclonal or antigen-specific stimulation. Evidence for preferential activation of Th1- or Th2-like populations in vivo has been obtained in animal and human systems (1–4, 7–9), most strikingly in parasite-induced responses. If, as hypothesized, this reflects preferential activation of Th1- or Th2-like patterns of cytokine synthesis, respectively, methods that would allow selective induction of specific patterns of cytokine gene expression could prove useful in manipulating de novo and established immune responses.

Depending on the reaction conditions selected, products with a wide range of immunological characteristics can be obtained after chemical modification of protein antigens with glutaraldehyde (10–13). Glutaraldehyde polymerized OVA (OA-POL), soluble OVA polymers of average relative molecular weight of 3.5×10^7, inhibits induction of IgE responses (14) and abrogates ongoing IgE synthesis (15) in a murine model of human immediate hypersensitivity. Support for the hypothesis that it acts by preferentially activating a Th1-like response is provided by the following observations: (*a*) CD4 T cell–dependent 95–99% decreases in OVA-specific IgE and 500–1,000-fold increases in IgG2a synthesis after administration of OA-POL but not unmodified OVA before (14) or after (15) OVA (alum) immunization; and (*b*) the demonstration that in vivo administration of anti-IFN-γ mAb XMG 1.2 neutralizes the effects of OA-POL on OVA-specific antibody responses (10). Such data provide strong, albeit indirect, evidence that chemical modification of antigen may be useful for induction of differential patterns of cytokine synthesis.

Here, we report that chemical modification of OVA can be used to deliberately and selectively elicit cytokine production characteristic of Th1-dominated responses. Whereas administration of unmodified OVA (intraperitoneally in saline) induces cytokine synthesis that is neither Th1 nor Th2 dominated, administration of OA-POL leads to preferential induction of Th1-like responses, as demonstrated by 20–23-fold increases in the ratio of IFN-γ/IL-4 or IFN-γ/IL-10 synthesis.

Materials and Methods

Treatment of Mice. C57BL/6 mice (6–12-wk-old) bred at the University of Manitoba breeding facility or purchased from Charles River Canada (St. Constant, Quebec, Canada), were used in accordance with guidelines issued by the Canadian Council on An-

imal Care. Mice tested negative for antibodies to mycoplasma, Sendai virus, and rodent coronaviruses including murine hepatitis virus by ELISA (Murine ImmunoComb; Charles River). OA-POL was prepared as described (10). Briefly, glutaraldehyde (6% in 0.15 M NaCl) was added dropwise to OVA (5× recrystallized; ICN Biochemicals, Montreal, Canada) at 25 mg/ml in 0.1 M acetate buffer (pH 5.3), 0.5 pH units above its isoelectric point, to yield a final molar ratio of 200:1. After dialysis and gel filtration (Biogel A-50m; Bio-Rad Laboratories, Mississauga, Ontario, Canada; $V_o = 5 \times 10^7$), OA-POL was recovered as a single, sharp, symmetric peak with an average relative molecular weight of 3.5×10^7. This method was developed in preference to previous approaches to glutaraldehyde modification which have been found to yield highly heterogeneous mixtures of reaction products exhibiting diverse immunological effects (11–13). OVA or OA-POL treatment consisted of three 80 μg injections (i.p.) on days 1, 3, and 5 before killing of the mice for culture on day 8. In light of the qualitative changes in cytokine production and antibody responses that are induced as a consequence of adjuvant administration (16, 17), all injections were carried out in saline, without adjuvants. Where used, anti-IFN-γ treatment consisted of injections of purified XMG 1.2 given as 250 μg/i.p. injection every day beginning at day −1, one day before initiation of OVA or OA-POL treatment, ending after day 5.

Cell Culture. In initial experiments, mice were killed 5–14 d after the initial administration of OVA or OA-POL. All of the experiments reported here were carried out 8 d after initial exposure to antigen, a time found to give maximal cytokine responses in the short-term culture system used. Spleen cell suspensions were cultured at 7.5×10^6/ml (2 ml/well) alone or with a predetermined optimal concentration of OVA (1 mg/ml) or ragweed extract (0.5 mg/ml, provided by Dr. A. Sehon, University of Manitoba, Winnipeg, Canada) in 24-well plates (Corning Science Products, Rochester, NY) at 37°C in complete medium (17). Duplicate cultures were established from the spleen cells of individual mice in each group. Culture supernatants were harvested for analysis of IL-4 and IFN-γ production at 20–24 h and IL-10 at 48 h.

Cytokine Determinations. IL-4 was determined as described (17), using a (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay to characterize the responsiveness of CT.4S cells to rIL-4 (provided by Dr. W. Paul, National Institute of Allergy and Infectious Diseases [NIAID], National Institutes of Health [NIH], Bethesda, MD) or sample tissue culture supernatants in the presence or absence of anti-IL-4 mAb 11B11 to assure specificity. IL-4-deficient medium was used as a negative control. As used here, this assay detects IL-4 at 0.15–0.30 U/ml and quantitates amounts ≥0.6 U/ml while being unresponsive to IL-2 levels below 100 U/ml. SE within any given experiment was usually <5%.

IFN-γ. A sandwich ELISA using purified anti-IFN-γ mAbs XMG 1.2 and purified, biotinylated R4-6A2 (American Type Culture Collection, Rockville, MD) in combination with streptavidin-alkaline phosphatase was carried out as previously described (17). Internal standards of IFN-γ–containing, Con A-stimulated mouse spleen cell supernatants, calibrated against World Health Organization-NIAID international reference reagent Gg02-901-533 (provided by Dr. C. Laughlin, NIAID, NIH), were included in each ELISA. The lower limit of detection is 0.2 U/ml for IFN-γ with quantitative measurement of cytokine ≥0.5 U/ml. SE was <10% in most experiments.

IL-10. A dual mAb-based ELISA with purified SXC1 (18) for antigen capture (1 μg/ml) and biotinylated-purified SXC2, streptavidin-alkaline phosphatase for development was used with IL-10 levels in culture supernatants determined against a rIL-10 standard. The lower limit of detection is 0.2 U/ml, with quantitation of cytokine ≥0.5 U/ml. SE was <10% in most experiments.

Statistical Analysis. Mean cytokine production ± SEM is shown. Statistical significance was determined using unpaired two-tailed Student's *t* tests.

Results and Discussion

Mice were administered unmodified OVA or OA-POL in saline and killed 8 d later, after which time short-term cultures were used to evaluate the pattern of cytokine response. Because cytokine production was examined after very short-term, antigen-specific in vitro stimulation directly ex vivo, we largely avoided the complications associated with clonal expansion or polyclonal activation (17) and were able to focus specifically on cytokine production by normal OVA-reactive cells.

As can be seen in Fig. 1, bulk culture of spleen cells obtained from such donors yielded readily detectable IL-4, IL-10, and IFN-γ responses after 20–48 h culture. In the absence of adjuvant, OVA elicited responses not dominated by Th1- or Th2-like cytokine gene expression patterns, as demonstrated by mean ratios of IFN-γ/IL-4 and IFN-γ/IL-10 synthesis of 1.23 and 1.07 in six and four experiments, respectively (Fig. 1). In marked contrast, cultures established from mice injected with OA-POL under the same conditions (intraperitoneally in saline) exhibited markedly reduced, OVA-driven IL-4 and IL-10 synthesis and increased IFN-γ production. Collectively, the ratio of antigen-stimulated IFNγ/IL-4 or IFNγ/IL-10 synthesis in groups given OA-POL was increased some 20-fold relative to that seen in cultures derived from groups given unmodified antigen (Fig. 1 *B*). These data provide direct evidence of preferential induction of a Th1-dominated cytokine response, at the population level, amongst OVA-reactive T cells after administration of appropriately modified antigen.

Tissue culture supernatants from cultures without antigen added in vitro usually yielded cytokine production below the level of detection. Cytokine synthesis above background was not obtained in cultures in which an unrelated antigen was added (ragweed extract, data not shown), demonstrating the antigenic specificity of the expression of these differential patterns of cytokine synthesis.

Several studies have demonstrated that the nature of cytokines present in the local environment at the time of T cell activation can strongly influence commitment to a particular pattern of cytokine synthesis (19–21). The data presented here suggested to us that early induction of IFN-γ production was responsible for driving commitment of the OVA-specific response towards a Th1-like pattern of cytokine gene expression. To better characterize the dependency of this commitment on in vivo IFN-γ synthesis elicited at the time of initial antigen exposure, mice were treated with unmodified OVA or OA-POL concurrent with anti-IFN-γ mAb. Animals were killed 8 d later and cytokine synthesis patterns were evaluated as above via overnight in vitro restimulation in the presence of OVA. As demonstrated in Fig. 2, concurrent in

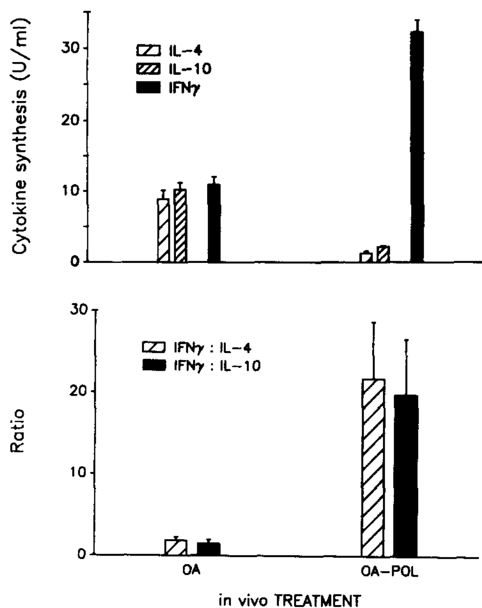

Figure 1. Relative production of Th1- and Th2-associated cytokines after in vivo administration of native or chemically modified OVA. (*A*) 8 d after intraperitoneal administration of OVA or OA-POL, cytokine synthesis was determined in 20–48-h, OVA-stimulated (1 mg/ml) bulk cultures as described in Materials and Methods. Data represent one of six independent experiments performed. (*B*) The ratio of IFN-γ/IL-4 or IFN-γ/IL-10 synthesis was determined for the cultures described above. Data represent mean ratios ± SEM of six independent experiments for IFN-γ/IL-4, four for IFN-γ/IL-10.

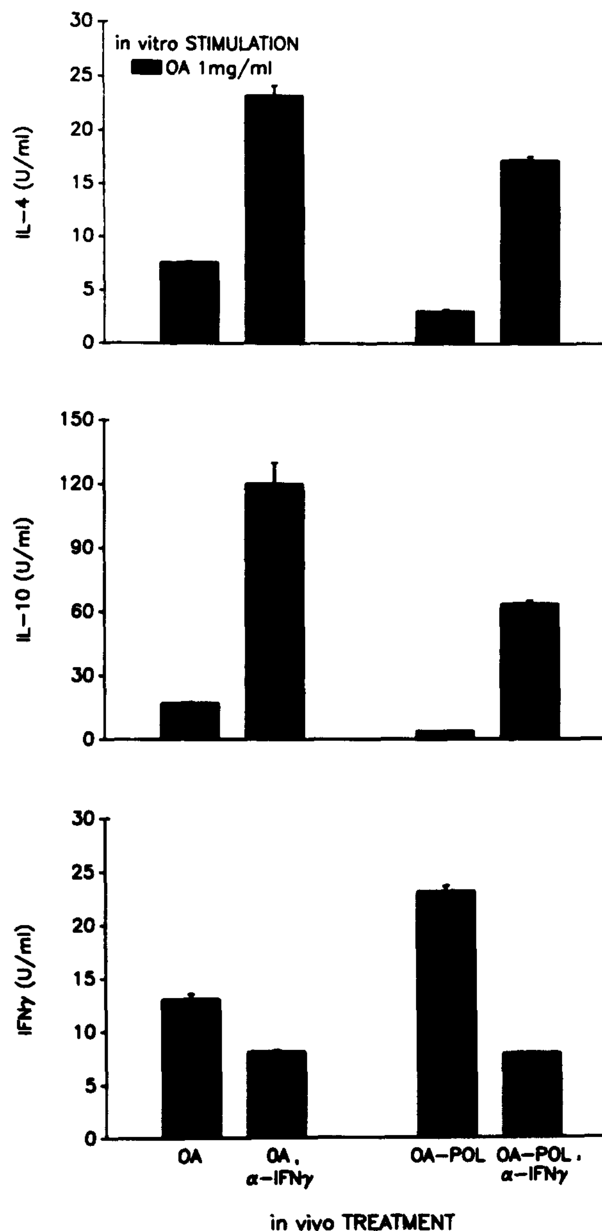

Figure 2. In vivo administration of anti-IFN-γ mAb concurrent with OVA or OA-POL treatment leads to induction of Th2-dominated responses. Cytokine production in 20–48 h. Supernatants of cultures established 8 d after treatment was determined as described in Materials and Methods.

vivo administration of anti-IFN-γ mAb and OA-POL results in marked increases in IL-4 and IL-10 production and decreases in IFN-γ production in response to antigen. This results in a reversal from a mean 8:1 ratio of IFN-γ/IL-4/10 synthesis in OA-POL-treated groups to a mean ratio of 0.26 after concurrent OA-POL/anti-IFN-γ treatment.

Concurrent anti-IFN-γ/OVA administration results in similar polarization of the response towards a Th2-dominated pattern of cytokine synthesis. Of interest was the observation that in vivo treatment with anti-IFN-γ treatment and OVA or OA-POL led to similar increases in IL-4 and IL-10 synthesis even in the absence of antigen in vitro (data not shown). Collectively, these data support the hypothesis that the balance of cytokine production that is elicited in the very early stages of antigen stimulation in vivo plays a pivotal role in determining the nature of the T cell response that ultimately comes to dominate the mature cytokine and effector responses to that antigen (19–22).

Whereas it remains unclear if commitment by normal individual T cells is as polarized in vivo as is commonly observed in long-term Th1 and Th2 clones, it is well established that certain antigens inherently induce dominantly Th1- or Th2-like responses in vivo (23, 24). In contrast, the present results demonstrate a capacity to selectively elicit either type of response by appropriate chemical modification of antigen.

The mechanism by which polymerized and unmodified OVA elicit qualitatively different patterns of cytokine synthesis remains unclear. Good evidence has been obtained that aqueous (unmodified) antigen administered in a protocol similar to that used here leads to dominance of a Th2 response resulting from clonal anergy of Th1 cells and IL-4–mediated crossregulation (25). Our finding that anti-IFN-γ treatment coadministered with OA-POL in vivo leads to marked increases in IL-4 production argues against direct anergy induction of Th2 cells in this system, and appears more consistent with preferential or positive selection of Th1 activity rather than negative selection or anergy induction in OVA-reactive Th2-like cells.

Particularly intriguing is the hypothesis that antigen presentation via B cells favors development of Th2-dominated

responses whereas macrophages support commitment to Th1 (26). We speculate that OA-POL, which is of very high relative molecular weight and exhibits 10–100-fold lower antigenicity than unmodified OVA, may induce a pattern of cytokine synthesis in vivo that is distinct from that seen after administration of native OVA because of minimal antigen presentation by OVA-specific B cells. In contrast, the very high relative molecular weight of OA-POL may enhance its processing by macrophages, contributing to a preferential activation of Th1-like activity. Experiments comparing the pattern of cytokine synthesis obtained in B cell–deficient (chronically anti-μ treated) mice after administration of native and chemically modified antigen are currently underway to address this issue.

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Address correspondence to Dr. K. HayGlass, Department of Immunology, University of Manitoba, Winnipeg, Canada R3E 0W3.

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