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Adjuvant-Guided Type-1 and Type-2 Immunity: Infectious/Noninfectious Dichotomy Defines the Class of Response¹

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Traditionally, protein Ags have been injected in CFA (oil with inactivated mycobacteria) to induce immunity and with IFA (oil alone) to induce tolerance. We report here that injection of hen eggwhite lysozyme, a prototypic Ag, in CFA-induced and IFA-induced pools of hen eggwhite lysozyme-specific memory T cells of comparable fine specificity, clonal size, and avidity spectrum, but with type-1 and type-2 cytokine signatures, respectively. This adjuvant-guided induction of virtually unipolar type-1 and type-2 immunity was observed with seven protein Ags and in a total of six mouse strains. Highly polarized type-1 and type-2 immunity are thus readily achievable through the choice of adjuvant, irrespective of the genetic bias of the host and of the nature of the protein Ag. This finding should have far-reaching implications for the development of vaccines against infectious and autoimmune diseases. Furthermore, our demonstration that Ag injected with IFA is as strongly immunogenic for T cells as it is with CFA shows that the presence of the mycobacteria determines not the priming of naive T cells through the second-signal link but the path of downstream differentiation toward CD4 memory cells that express either type-1 or type-2 cytokines. *The Journal of Immunology*, 1999, 162: 3942–3949.

Type-1 and type-2 immunity involve fundamentally different and opposing effector functions.³ The induction of a type-2 response can be deleterious to the host when a type-1 response would be protective and vice versa; therefore, successful vaccination depends critically on the ability to induce the desired class of response selectively and reliably.

Purified protein Ags and peptides, the currently favored subunit vaccine candidates, are poor immunogens. Injection of these sub-

stances usually induces immunological tolerance unless administered with an adjuvant (6–9), traditionally CFA. This generally known yet poorly understood need to contaminate pure Ags with mycobacteria to induce immunity has been called the “immunologist’s dirty little secret” (10). To explain this and similar observations made over the decades, models that tie the induction of either an immune response or tolerance to the activation state of the innate immune system have been proposed (11, 12). According to these models, microbial constituents and other signals recognized as “infectious nonself” or as “danger” activate professional APCs, which causes them to acquire costimulatory properties. The “second signal” required for T cells to be primed (13, 14) is delivered by activated APC; Ag encountered on resting APC inactivates T cells, thus accounting for naturally developing as well as experimentally induced tolerance. Infectious agents are thought to be the prime candidates causing activation of APC (reviewed in Refs. 15 and 16). The classic Ag injection protocols with CFA or IFA seemed to support this hypothesis. Inoculations with Ag in CFA (oil containing inactivated mycobacteria) has been thought to be the most forceful way of inducing an immune response to protein Ags (6), while the injection of Ag in IFA (oil alone) has been thought to induce tolerance, particularly when done i.p. (17–19).

While revisiting neonatal Ag:IFA injections, we observed that this method of administering Ags does not induce immunological tolerance but triggers a vigorous type-2 immune response (20). These data have since been confirmed by several groups (21–23). Because neonates are thought to be biased toward type-2 immunity (24, 25), we performed the experiments presented here to test stringently the hypothesis that the supposedly tolerance-inducing Ag:IFA injections would also trigger type-2 immunity in adult mice. Having found this to be the case and because of the rigor that would be required if a classic paradigm were to be redefined, we decided to characterize closely the immune response induced. We

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³ Note to type-1/type-2 classification: Presently, it is controversial whether the T cells that mediate type-1 and type-2 responses in vivo secrete mutually exclusive sets of type-1 and type-2 cytokines (Th1/Th2 model) (1), whether they randomly coexpress these cytokines in a continuous range of concentrations (stochastic model) (2), or whether each T cell produces only a single cytokine (3). To avoid these uncertainties of cytokine coexpression in individual memory T cells in vivo, we purposefully chose the type-1/type-2 terminology, which describes types of in vivo responses (4, 5), instead of the Th1/Th2 nomenclature (1), which implies cytokine expression patterns in the individual memory T cells that mediate these responses. We used the term type-1 to describe the production of IFN- γ ⁺, IL-5⁻ by memory T cells, which mediate delayed-type hypersensitivity and promote specific IgG2a but not IgE production. Type 2 responses were defined as IFN- γ ⁻, IL-5⁺ production in memory T cells, which do not mediate delayed-type hypersensitivity but promote IgE and IgG1 production in the absence of IgG2a.

showed that hen eggwhite lysozyme (HEL)⁴ is equally immunogenic when injected in CFA and IFA in that it induces T cell responses of comparable clonal size, fine specificity, and avidity but differing in cytokine profile, being type-1 and type-2, respectively. Moreover, we show that these results may be generalizable as they were seen with seven protein Ags and in a total of six mouse strains. We therefore conclude that it is not the “infectious/noninfectious” or “dangerous/nondangerous” character of the immune challenge that provides the “second signal” determining whether T cell immunity or T cell tolerance is induced. Instead, this dichotomy seems to determine whether T cells differentiate along the type-1 or type-2 pathway.

Materials and Methods

Mice, Ags, and injections

Mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and bred at Case Western Reserve University under specific pathogen-free conditions. Female mice were injected at 6–10 wk of age. HEL, OVA, and BSA were purchased from Sigma (St. Louis, MO). Guinea pig myelin basic protein (MBP), rabbit renal tubular Ag (RTA), *Schistosoma* egg Ag (provided by C. King), and *Leishmania* Ags were prepared as described (26–28). The peptides OVA:323–339 (KISQAVHAAHAEINEAG), HEL:106–116 (NAWVAVRNCRCK), and proteolipid protein peptide (PLP) PLP:140–152 (HCLGKWLGHDPDKF) were purchased from Princeton Biomolecules (Columbus, OH). IFA was purchased from Life Technologies (Grand Island, NY), and CFA was made by mixing *M. tuberculosis* H37RA (Difco Laboratories, Detroit, MI) at 2.5 mg/ml into IFA. Alum (Imject Alum) was purchased from Pierce (Rockford, IL). Individually, Ags or peptides were mixed with the adjuvant to yield a 2-mg/ml emulsion, of which 50 μ l (100 μ g/mouse) was injected once, i.p. or s.c., as specified.

Proliferation assays

Spleen cell proliferation assays were performed as previously described (29). Briefly, single-cell suspensions were prepared, and 1×10^6 spleen cells (single-cell suspensions that had not been depleted of erythrocytes) were plated per well in flat-bottom, 96-well microtiter plates in serum-free HL-1 medium (BioWhittaker, Walkersville, MD) supplemented with L-glutamine at 1 mM. Ags were added at a final concentration of 100 μ g/ml. During the last 18 h of a 4-day culture, [³H]thymidine was added (1 μ Ci/well); incorporation of label was measured by liquid scintillation counting. The lymph node (LN) assays followed the same protocol except that 5×10^5 cells were plated per well.

Cytokine ELISPOT assays

The use of plates and of an image analyzer customized for ELISPOT assays greatly improved the resolution and sensitivity of this assay to the extent that it has single-cell resolution and is suited to measure cytokine production of low-frequency Ag-specific memory T cells in freshly isolated cellular material (20). Briefly, plates (ImmunoSpot, Resolution Technology, Columbus, OH) were coated overnight at 4°C with the cytokine-specific capture Abs specified below. The plates were then blocked with 1% BSA in PBS for 1 h at room temperature and washed four times with PBS. Subsequently, the Ags, peptides, and freshly isolated single-cell suspensions of spleen or LN cells were added under the conditions specified for proliferation assays. After 24–48 h of cell culture in the incubator, the cells were removed by washing three times with PBS and four times with PBS containing 0.05% Tween (PBST). Then, the detection Abs were added and incubated at 4°C overnight (the detection Abs were either horse radish peroxidase (HRP)-labeled or biotinylated). The plates were washed three times with PBST. If biotinylated detection mAbs were used, streptavidin-HRP conjugate (Dako, Carpinteria, CA) was added at 1:3000 dilution, incubated for 2 h at room temperature, and removed by washing twice with PBST and twice with PBS. The spots were visualized by adding HRP substrate 3-amino-9-ethylcarbazole (Pierce, Rockford, IL). We used the following combinations of capture and detection mAbs for IL-2, IL-4, IL-5, and IFN- γ assays respectively: JES6-1A12 (5 μ g/ml) and JES6-5H4-biotin (2 μ g/ml), BVD4-1D11 (2 μ g/ml) and BVD4-24G2-biotin (2.5 μ g/ml), TRFK5 (5 μ g/ml) and TRFK4-HRP (2 μ g/ml), and R46A2 (5 μ g/ml) and XMGI.2-HRP (2 μ g/ml). Image analysis of ELISA spot assays was per-

formed on a Series 1 ImmunoSpot Image Analyzer (Resolution Technology, Columbus, OH) customized for analyzing ELISA spots to meet objective criteria for size, chromatic density, shape, and color. To evaluate the results in the HEL system, we used image analysis; some of the results summarized in Table I were obtained before we had access to an image analyzer and so were counted visually.

Measurement of specific serum Abs

Plates (Nunc Immoplate, Fisher Scientific, Pittsburgh, PA) were coated with HEL (10 μ g/ml) overnight at 4°C, then blocked for 1–2 h with 0.1% gelatin, both in PBST. The test serum was added and incubated overnight at 4°C. Plate-bound Ab was detected by alkaline-phosphatase-coupled anti-mouse Ig. Affinity-purified goat anti-mouse IgG(H+L) from Southern Biotechnology (Birmingham, AL) was used to detect total Ig; the isotype-specific Abs used to detect IgG1 and IgG2a were also from Southern Biotechnology. *p*-Nitrophenyl phosphate disodium salt (Research Organics, Cleveland, OH) was used for the development of the colorimetric reaction.

Determinant mapping with overlapping peptides

The 118 HEL 12-mer peptides that covered the entire sequence of the HEL molecule in single steps of amino acids (30) were purchased from Chiron Mimotopes (Raleigh, NC). Peptides were dissolved at 560 μ M in pH-optimized buffer (either 0.2 M HEPES or 0.2 M acetic acid), aliquoted into 96-well microtiter plates, and stored frozen at –85°C. Freshly thawed peptides were used in the cytokine ELISPOT assay at 7 μ M.

Cell separations

Animals were injected with Ag in adjuvant, and 3–4 wk later single-cell suspensions were prepared from their spleens. Erythrocytes were depleted by Ficoll density gradient separation. CD4 cells were obtained by passing erythrocyte-depleted spleen cells through a murine CD4⁺ T cell enrichment column (R & D Systems, Minneapolis, MN) and further separated using Dynabeads (Dyna, Lake Success, NY) to obtain L-selectin⁺ and L-selectin[–] cells. FACS analysis was performed after each fractionation step and showed >90% enrichment for the desired phenotype (as specified in the figure legends for the experiments shown). All cell fractions were plated at 2×10^5 cells/well and tested in proliferation and ELISPOT assays with irradiated BALB/c SCID APCs in the presence or absence of HEL at 100 μ g/ml or anti-CD3 mAb 2C11 (American Type Culture Collection, Manassas, VA) at 1 μ g/ml in HL-1 medium. The bioassays were performed as specified above.

Results

Intraperitoneal injection of Ag in IFA induces CD4⁺, L-selectin[–] proliferative memory in the spleen but not in nondraining LN

Spleen cells of mice injected i.p. with HEL:IFA showed a vigorous Ag-specific proliferative recall response of a magnitude comparable to that induced by s.c. HEL:CFA immunization (Fig. 1). Specific proliferative memory responses were also detected after i.p. IFA injections of other protein Ags in several mouse strains (data not shown, summarized for cytokine recall in Table I), indicating that Ag i.p. injected in IFA induces an immune response rather than immunologic tolerance.

After i.p. injections, no recall responses were detected in peripheral LN (popliteal or brachial LN, which do not drain the peritoneal cavity) of mice whose spleen cells responded vigorously (Fig. 2A). This lack of response in nondraining LN was seen after all i.p. injections, irrespective of the adjuvants used, mouse strains tested, or harvest times after injection (1–8 wk). Similarly, mice injected s.c. with HEL:CFA yielded recall responses in the draining popliteal LN but not in the nondraining brachial LN (Fig. 2B), the latter of which may have been caused by the down-modulation of the LN homing receptor (L-selectin) on memory cells (31, 32). To test this hypothesis, we separated subpopulations of primed spleen cells and showed that the responding cells were L-selectin[–], CD4⁺ cells (Fig. 2, C and D). The

⁴ Abbreviations used in this paper: HEL, hen eggwhite lysozyme; MBP, myelin basic protein; RTA, renal tubular Ag; PLP, proteolipid protein; LN, lymph node; PBST, PBS with Tween; HRP, horseradish peroxidase; SI, stimulation index.

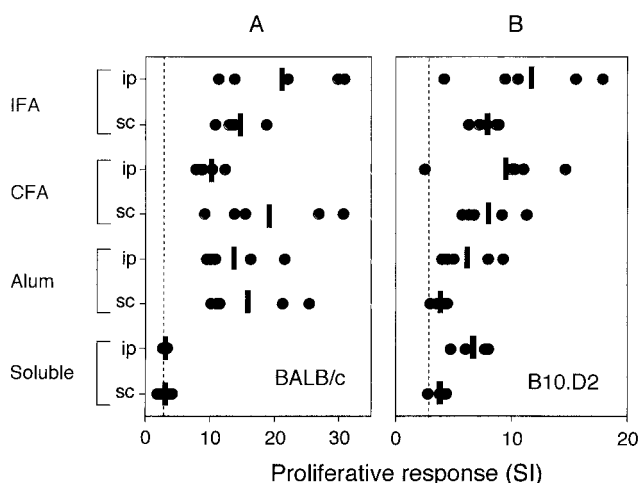


FIGURE 1. The proliferative recall response in spleen cells of HEL-injected mice. BALB/c (A) and B10. D2 (B) mice were injected with 100 μ g HEL in IFA, CFA, alum, and saline, s.c. and i.p., as indicated. Three weeks later, the proliferative recall response was tested. The results are expressed for individual mice as stimulation indices (SI = mean cpm of triplicate HEL-containing wells divided by mean cpm of triplicate wells with medium alone; the medium control ranged between 200 and 3000 cpm for individual mice with no significant differences in background proliferation among the groups). Dashed lines show SI = 3, the margin between positive and negative results. The HEL-primed spleen cells did not proliferate in the presence of the control Ag (MBP), and MBP-primed spleen cells did not proliferate to HEL but did to MBP, which confirms the Ag specificity of the recall response (data not shown). Data shown were obtained in a single experiment and are representative of three performed with HEL.

lack of LN-homing-receptor expression may explain why few memory cells migrated to nondraining LN and, thus, why the previous studies, which focused on measuring T cell responses in LN, missed the fact that immunity rather than tolerance had been induced after the i.p. injections.

Intraperitoneal IFA injection induces type-2 cytokine memory

The fact that memory cells occur at low frequencies (19, 33) has made it challenging to obtain accurate measurements of the cytokine recall responses of freshly isolated T cells to single nominal Ags such as HEL. To overcome this limitation, we developed a modification of the cytokine ELISPOT assay that can detect and accurately measure the frequency of single cytokine-producing cells.⁵ Using this assay, we have characterized the cytokine signatures of the T cell responses induced by Ag injections in IFA and CFA.

Freshly isolated spleen cells from i.p. HEL:IFA-injected mice produced 20–100 IL-5 spots per million spleen cells when cultured with HEL for 48 h; fewer than three spots (usually none at all) were seen in cultures containing medium alone (Fig. 3), which yields a stimulation index (SI) ranging from 7 to 100. This IL-5 was produced by CD4⁺ T cells, and when challenged with HEL these CD4⁺ cells also produced comparable numbers of specific IL-4 and IL-2 spots (data not shown), but no HEL-specific IFN- γ production was seen (Fig. 3, <8 spots, SI = 1). In contrast, in mice injected s.c. with HEL:CFA, a specific IFN- γ response of comparable magnitude was recalled (SI = 40–90) with no IL-5 production in the spleen (Fig. 3). No IL-5 or IL-4 spots were induced by

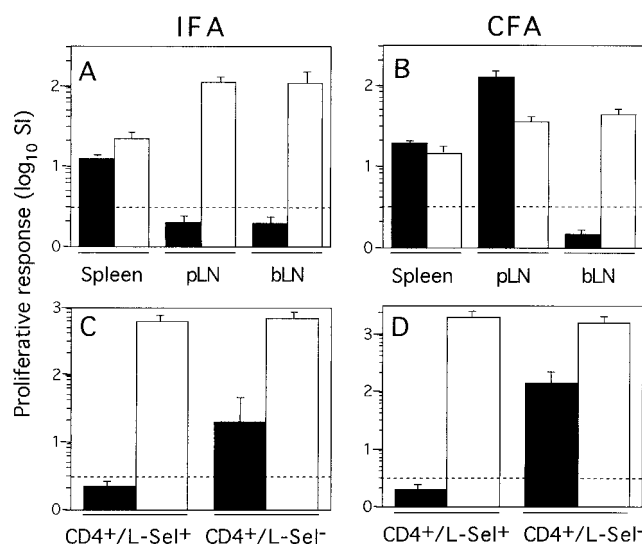


FIGURE 2. Tissue distribution of memory cells induced by HEL:IFA and HEL:CFA. A, Lack of proliferative recall response in nondraining LN of i.p. HEL:IFA-injected BALB/c mice whose spleen cells responded. Three weeks after the injection, pooled spleen cells, popliteal LN cells (pLN), and brachial LN cells (bLN) (both nondraining LN) were tested in a proliferation assay for their response to HEL (closed bars) and the anti-CD3 mAb-induced response (open bars). We could not test the mediastinal postthymic LN, which drain the peritoneal cavity, because these LN were too small to identify. The mesenteric LN of these mice did not yield a recall response (data not shown). B, Recall response in the draining LN but not in nondraining LN after s.c. CFA immunization. BALB/c mice were immunized with HEL:CFA, s.c., and their draining (pLN) and nondraining (bLN) LN were tested. The data are representative of systematic studies showing that the response in the spleen stayed at constant positive levels for up to 1 year but while that in nondraining LN remained negative. C and D, Phenotype of the memory cells in the spleen after i.p. HEL:IFA (C) or i.p. HEL:CFA (D). Three weeks after the injection, spleen cells of six BALB/c mice were pooled, and CD4⁺ cell fractions were obtained by negative selection and further subfractionated into L-selectin positive and negative populations as described in *Materials and Methods*. In the experiment shown, enrichment for the CD4 marker resulted in 99% purity, and subsequent depletion of L-selectin⁺ cells resulted in a 98% L-selectin⁻ population. The HEL-specific proliferative response of these cell fractions was tested using *H-2-congenic SCID* APC in a proliferation assay as specified in *Materials and Methods*. Results are expressed as mean \pm SD of triplicate wells. Dashed lines show SI = 2. The data are representative of two experiments performed with the same result. All the data in this figure have also been reproduced in cytokine ELISPOT assays, the results of which supported the same conclusions: memory cells do not efficiently migrate to nondraining LN because L-selectin has been down-regulated.

HEL in the draining LN of such mice, but apparently Ag-specific IL-4 production was seen in the spleen (however, cell separation experiments showed that this IL-4 was not derived from T cells, but was induced as a cytokine-mediated bystander reaction in APC; A.Y.K, manuscript in preparation). In both the type-1-biased B10.D2 and the type-2-biased BALB/c strains (34), injection of HEL in either IFA or CFA induced virtually unipolar cytokine recall responses (Fig. 3). Similar results were obtained with six mouse strains of mixed type-1 and type-2 biases and six protein Ags other than HEL (Table I). Even proteins with well-defined intrinsic type-1/type-2-polarizing properties such as *Leishmania* and *Schistosoma* egg Ags (35, 36) followed this pattern. These data suggest that, for protein Ags, the choice of adjuvant (facilitated by the route of injection, see below) might suffice to override

⁵ A. Y. Karulin, M. D. Hesse, M. Tary-Lehmann, and P. V. Lehmann. Direct visualization of cytokine coexpression by individual in vivo differentiated CD4 cells. Submitted for publication.

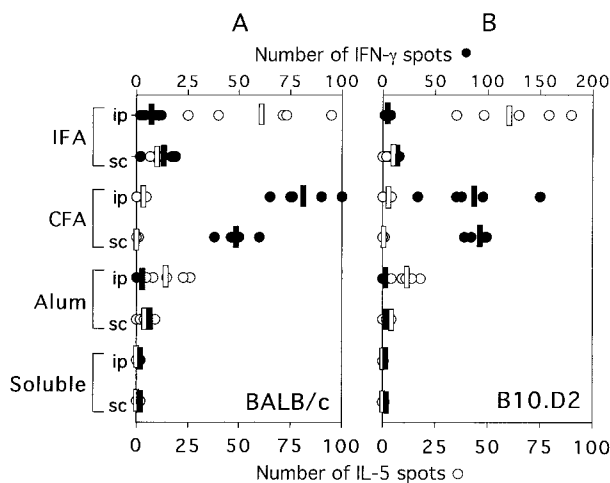


FIGURE 3. The cytokine recall response in spleen cells of HEL-injected mice. BALB/c (A) or B10.D2 (B) mice were immunized as indicated and a cytokine ELISPOT assay was performed on freshly isolated spleen cells 3 wk later. The frequency of cells producing IFN- γ (closed circles) and IL-5 (open circles) per million cells plated is shown for individual mice, with the mean indicated by the bars. Cytokines were produced only in the presence of HEL; wells with medium and control Ag (MBP) contained less than five cytokine spots each. The spots were counted by a computerized image analysis system (SD among replicate wells was <20%). The data have been reproduced in three experiments. These results are representative of data from a wide range of time points after immunization (2 wk to 6 mo).

both the type-1 and type-2 biases of the hosts and Ags. This observation, which we first reported for a prototypic “foreign” and “self” Ag (HEL and MBP) in two strains of mice (BALB/c and

B10.PL) at neonatal and adult ages (20), seems, therefore, to be generalizable to adult mice (and neonatal mice as well; T.F., manuscript in preparation).

We have tested three MHC class II-restricted peptides so far (Table I). PLP peptide 140–152 in SJL mice and HEL peptide 106–116 in DBA/2 mice behaved as did the protein Ags. Although the injection of the third peptide, OVA:323–339, in CFA, into BALB/c mice also induced a unipolar IFN- γ response, when injected in IFA it triggered mixed IFN- γ :IL-5 memory (Table I). Why the response to this OVA peptide was type-1 biased is unclear. Unlike proteins, peptides can directly bind to MHC molecules on the cell surface, reaching MHC/peptide densities that would not occur after intracellular processing of the native protein. Such a high ligand density might have favored type-1 differentiation (37, 38).

Intraperitoneal HEL:IFA injections induce specific IgG1 but not IgG2a production

We chose to use HEL, one of the best-characterized protein Ags, to define the adjuvant-induced immune responses more closely. Mice injected i.p. with HEL:IFA produced specific IgG1 but did not produce detectable levels of IgG2a Abs (Fig. 4). Because switching of Ig isotype to IgG2a is IFN- γ dependent (39), this finding supports the notion that IFA induces Th cells that do not produce IFN- γ . In CFA-immunized mice, IgG2a was produced, though IgG1 was also. This coexpression of IgG1 and IgG2a is not surprising because switching to IgG1 is upstream of IgG2a and is not strictly IL-4-dependent, but can also be promoted by IL-2 (40, 41). Therefore, specific IgG1 Abs may be a less reliable surrogate marker for type-2 immunity than generally anticipated. We found that the sera of HEL:CFA-immunized BALB/c mice tested negative for IgE in specific, passive cutaneous anaphylaxis assays, confirming previous findings (42), while sera of HEL:IFA-injected

Table I. Cytokine recall response in spleens of mice injected with various Ags

Strain	No. of Mice	Ag ^a	Adjuvant and Route	Cytokine Recall Response ^b (no. of cytokine-producing cells/10 ⁶)	
				IL-5	IFN- γ
C57.BL/6	8	OVA	IFA i.p.	40 \pm 23	<3
	8	OVA	CFA i.p.	<3	82 \pm 12
	4	<i>Leishmania</i>	IFA i.p.	19 \pm 3	<3
	4	<i>Leishmania</i>	CFA i.p.	<3	31 \pm 5
BALB/c	4	BSA	IFA i.p.	9 \pm 3	<3
	4	BSA	CFA i.p.	<3	20 \pm 12
	4	BSA	CFA s.c.	<3	42 \pm 20
	4	<i>Leishmania</i>	IFA i.p.	21 \pm 4	<3
	4	<i>Leishmania</i>	CFA i.p.	<3	19 \pm 3
	4	<i>Schistosoma</i>	IFA i.p.	58 \pm 33	<3
	4	<i>Schistosoma</i>	CFA i.p.	<3	40 \pm 24
	24	OVA	IFA i.p.	43 \pm 10	<3
	24	OVA 323-339	IFA i.p.	40 \pm 1	56 \pm 15
	24	OVA 323-339	CFA s.c.	<3	71 \pm 15
SJL	3	RTA	IFA i.p.	55 \pm 21	<3
	3	RTA	CFA s.c.	<3	66 \pm 20
	5	PLP 140-152	IFA i.p.	14 \pm 8	<3
	6	PLP 140-152	CFA s.c.	7 \pm 6	50 \pm 21
	5	MBP	IFA i.p.	18 \pm 3	<3
B10.PL	17	MBP	IFA i.p.	25 \pm 15	<3
	24	MBP	CFA i.p.	<3	46 \pm 8
	5	MBP	CFA s.c.	<3	73 \pm 28
B10.D2	4	BSA	IFA i.p.	24 \pm 15	<3
	4	BSA	CFA i.p.	<3	30 \pm 16
	4	BSA	CFA s.c.	<3	35 \pm 28
DBA/2	2	HEL 106-116	IFA i.p.	29 \pm 4	7 \pm 2
	2	HEL 106-116	CFA s.c.	<3	39 \pm 2

^a A total of 100 μ g of each Ag was injected as described in *Materials and Methods*, with the exception of *Leishmania*, in which 50 μ g was used.

^b Cytokine ELISPOT assays were performed on spleen cells 2–6 wk after injection. The responses were specific for the Ag injected. Mean \pm SD for all mice in the groups are shown. Legend to Fig. 3 applies.

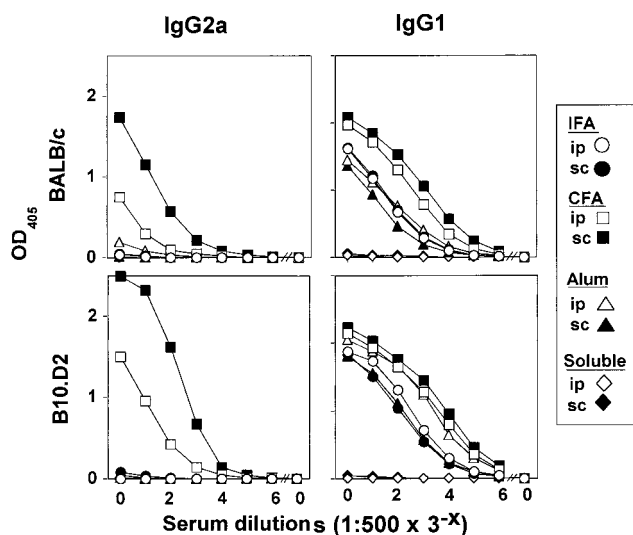


FIGURE 4. Specific serum Ab isotypes in HEL-injected mice. BALB/c (top) or B10.D2 (bottom) mice were injected with HEL as specified by the symbols on the right, six mice per group. Three weeks later, the mice were bled and their HEL-specific IgG2a and IgG1 serum Ab titers were measured by ELISA. The results shown are mean values of groups of six mice and are fully representative for the individual mice tested. The titration shown started at serum dilution 1:500 and progressed in steps of 1:3 with each dilution shown; the last data point represents results obtained from serum of an unimmunized mouse diluted 1:500. Error bars would have fallen within the size of the symbols. The results are representative of three experiments performed.

mice tested positive (data not shown). Because IgE Abs are IL-4-dependent (41, 43, 44), their absence provided further evidence that the memory T cells induced by the CFA immunization did not produce IL-4 (and that the IL-4 bystander reaction detected in the spleens of such mice might have limited functional relevance; see Footnote 3).

Alum, like IFA, is a type-2 adjuvant that activates the same magnitude of humoral, but a weaker level of cellular, type-2 immunity

Having observed unexpected type-1 and type-2 polarization when using Freund's classic adjuvants, we decided to include alum in our studies as it is the only adjuvant presently in general use for human vaccinations (9). Injection of HEL in alum, both s.c. and i.p., resulted in the production of IgG1 but not IgG2a Abs comparable in magnitude to the IFA-induced response (Fig. 4); however, significant cytokine recall responses were seen only after i.p. alum injections (Fig. 3). These were unipolarly IL-5⁺:IFN- γ ⁻ but involved an overall frequency of IL-5-producing cells fivefold lower than that after i.p. IFA injections. These data suggest that, although both alum and IFA promote type-2 responses, they differ in their propensity to engage the cellular, as opposed to the humoral, effector arm of type-2 immunity; IFA is more proficient at driving the clonal expansion of IL-5-producing memory cells.

With IFA and alum, the i.p. route facilitates generation of a type-2 cellular response

The clonal size of Ag-specific IL-5-producing cells induced by i.p. HEL:IFA injection was ~ 10 -fold greater than that seen after s.c. administration of HEL:IFA (Fig. 3). Moreover, the weak cytokine recall response engaged using the s.c. route included some IFN- γ -producing cells in addition to the IL-5 producers (Fig. 3). With alum, the i.p. route also facilitated the generation of IL-5-producing

memory cells as with IFA. In contrast to the cytokine response, the Ab levels induced by i.p. and s.c. injections with either IFA or alum were of a comparable titer and had a pure IgG1⁺ and IgG2a⁻ profile (Fig. 4). Apparently, the relatively few type-2 memory cells primed by s.c. injection were sufficient to provide maximum help toward IgG1 Ab production even though the i.p. route facilitated the generation of ~ 10 times more type-2 memory cells. Because the immune response occurs in LN associated with either systemic or mucosal immunity when the Ag is injected s.c. (popliteal LN) or i.p. (mediastinal postthymic LN), respectively, different lymphoid tissues are involved when the Ag is injected s.c. and i.p., and the differences associated with the route of immunization might be the result of differences in the influence of local cytokines (45) or hormones (46).

Anti-HEL T cell responses induced by i.p. IFA and CFA injections have identical fine specificities

Our finding, that i.p. injection of Ag induces type-2 immunity may explain why some earlier reports implied that this protocol induces tolerance by activating suppressor cells. Because fine specificities that differ from those of CD4⁺ cells have been attributed to suppressor cells (47), we mapped the anti-HEL response after i.p. IFA and CFA immunization using a complete HEL peptide series that covered every possible determinant on the molecule in steps of single amino acids. The data showed that identical T cell specificities were engaged in both instances (Fig. 5, A and B), leading to the recognition of region amino acid 105–119 (core: 108–116), located within the well-defined, I-E^d-restricted determinant, 106–116 (30). The recognition of this MHC class II-restricted determinant suggested that CD4⁺ cells had been induced, confirming the results of the cell separation experiments shown in Fig. 2.

Cytokine responses induced by IFA and CFA have comparable dose response characteristics.

One possible interpretation of our data was that i.p. HEL:IFA injections deleted T cell clones with high avidity for the Ag (48) and that the type-2 response measured after this treatment represented residual activity from low-avidity T cells. To address this possibility, spleen cells from BALB/c mice were injected i.p. with either HEL:IFA or HEL:CFA, and the responses were recalled in ELISPOT assays in which the immunodominant peptide HEL: 106–116 was titrated. Following the classic definition of functional avidity for polyvalent ligand-receptor interactions, functional T cell avidity can be defined as the concentration of peptide that leads to the activation of 50% of T cells in a given population for a constant number of APCs (49). T cells become activated (a process that includes the induction of cytokine production) when a critical number of MHC-peptide ligands are bound by their TCR (50). This number depends on the intrinsic affinity of the TCR for the particular MHC-peptide complex (51): high-avidity T cell clones will be activated at low peptide concentrations and those of low avidity at high concentrations. Because the frequencies of cytokine-producing T cells measured by ELISPOT in the cells primed with CFA and IFA had similar dose-response characteristics (Fig. 5, C and D), we can conclude that the T cells primed with CFA and IFA had comparable functional avidities. These data suggest that similar precursor-cell repertoires, including the high-avidity end of the spectrum, were primed by both immunizations.

Discussion

There are several reasons, primarily historic, that Ag:IFA injections were thought to induce T cell tolerance rather than immunity. First, T cell immunity was initially measured either by transplant

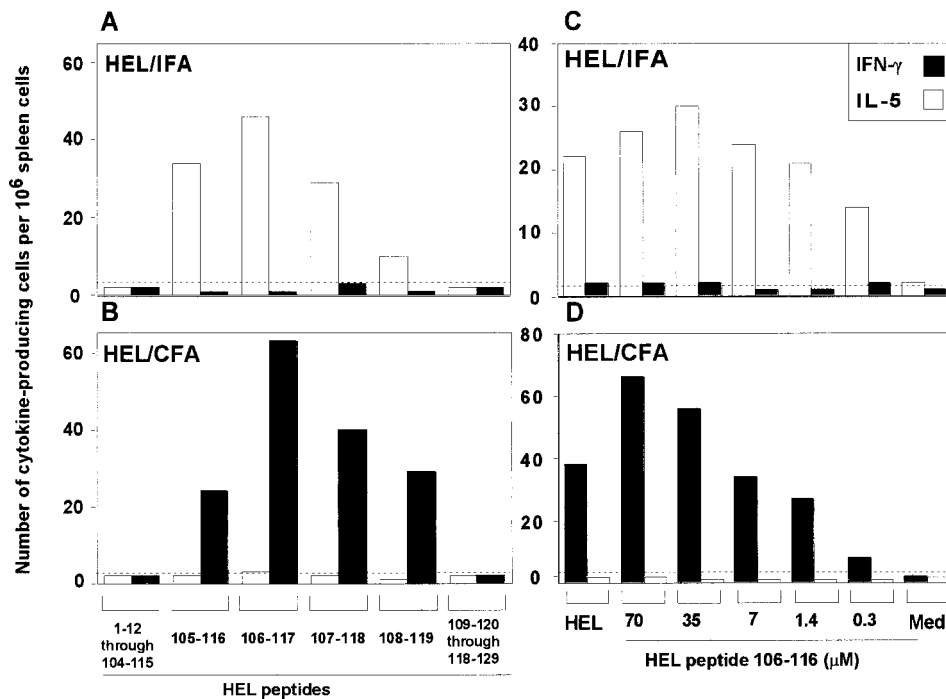


FIGURE 5. Fine specificity of the responses induced by i.p. HEL:IFA and i.p. HEL:CFA injection (*A* and *B*) and their functional avidities (*C* and *D*). Six adult BALB/c mice in each group were injected and their spleen cells were pooled and tested in IL-5 and IFN- γ ELISPOT assays for HEL peptide responses 3 wk later. In *A* and *B*, we tested all 118 peptides at 7 μ M, covering the entire HEL sequence in steps of one amino acid. Wells with peptides 1–12 through 104–115 and 109–120 through 118–129 contained less than three IL-5 or IFN- γ spots each and are therefore represented in the single bars. None of the peptides induced cytokine spot formation over background in uninjected or in control-injected mice (<3 spots per well, dashed lines, data not shown). The results for peptides 105–116 to 108–119 are from single wells of one experiment of two performed with identical results. In *C* and *D*, the dose-responses to HEL peptide 106–116 was tested. For mice immunized with either HEL:IFA or HEL:CFA, HEL protein at 7 μ M and HEL:106–116 at 35–70 μ M recalled the maximum response and at 0.06 μ M failed to induce a response over that of the medium control shown. The variations between the four replicate wells was <20%. The data are representative of two experiments performed.

rejection, by the development of T cell-mediated autoimmune diseases such as experimental allergic encephalomyelitis or by delayed-type hypersensitivity, all of which are manifestations of type-1 T cell effector functions and neither of which is induced by Ag in IFA (7, 8). Second, while several groups noted early on that the IFA-induced “tolerance,” as measured by the prevention of experimental allergic encephalomyelitis and delayed-type hypersensitivity, can be adoptively transferred (52–54), this infectious tolerance was interpreted according to the prevalent models of the time as evidence for the induction of suppressor cells. Today these data can be interpreted as type-2 regulation of type-1 effector functions. Third, until the early 1990s, CD4⁺ T cell responses could only be measured in draining LN using proliferation assays (55); treatments that abrogated such responses were thought to induce tolerance. As we have shown (Fig. 2), memory T cells primed after i.p. immunization are LN-homing-receptor-negative and do not migrate efficiently to LN, though they are present in the spleen. This compartmentalization of memory T cells is consistent with the present understanding of T cell migration patterns (56–59). Fourth, proliferative recall assays that detect CD4⁺ memory in the spleen became feasible only with the advent of serum-free assay conditions, at which time attention was drawn to the detection of CD4⁺ memory cells in the spleen (29). Finally, measurements of the cytokines produced by Ag-specific cells have been challenging, particularly for type-2 cytokines. Performing ELISAs on culture supernatants and ELISPOT assays in parallel, we found that between 200 to 400 T cells had to produce IL-4 and IL-5 before these cytokines could be detected by ELISA in the culture supernatants. This relatively low sensitivity might be why the immunogenicity

of Ag:IFA injections was not detected, even in TCR transgenic adoptive transfer models; however, there are several earlier reports according to which s.c. IFA injections induce “humoral” but not “cell-mediated” (type-1) immunity (7, 8, 60).

Our finding that i.p. IFA injections are strongly immunogenic might provide new insights into what is required to induce T cell responses in general and type-1 and type-2 immunity in particular. It has generally been held that to promote a strong immune response an adjuvant must simultaneously perform two critical functions (reviewed in Refs. 7 and 8). First, the adjuvant must function as an Ag depot. The oil-based Freund’s adjuvants excel at retaining and slowly releasing Ag; the half-life of OVA in IFA is ~90 days (60). Second, it is now widely accepted that adjuvants must locally activate APC to engage the “second signal,” a reaction that is thought to be primarily induced by microbial products (reviewed in Refs. 15 and 16). Because we show that IFA is as strong an adjuvant for inducing type-2 immunity as CFA is for type-1 responses, the “infectious nonself” hypotheses might have to be revised in favor of one of the following models.

Model 1 states that the activation of the innate immune system by “infectious nonself” signals is required for the induction of type-1 but not type-2 immunity. In this model, “infectious nonself” might correspond to the up-regulation of B7-1 molecules and/or the induction of IL-12. When cells of the innate immune system, which include dendritic cells, are exposed to certain microbial products that these cells have evolved to recognize as common features of “foreign,” such as carbohydrates (reviewed in Ref. 16), they up-regulate B7-1 and secrete IL-12, resulting in a microenvironment that favors T cell differentiation along the type-1

pathway (61, 62). In contrast, type-2 immunity may develop by default when T cells encounter Ag on “unstimulated” APC (which in a normal microbial environment may constitutively express enough costimulatory molecules, including B7-2, to activate T cells). This view might be substantiated by an increasing number of recent reports of soluble protein Ags inducing type-2 immunity when administered epicutaneously (63), orally (64), nasally (65), and i.v. as aggregated proteins (66). The fact that HEL:IFA promotes a stronger T cell response than does HEL:alum while the same dose of soluble HEL is barely immunogenic might merely reflect the unique Ag-retaining properties of IFA. In this scenario, the role of IFA as an adjuvant is passive, limited to serving as an Ag depot (60).

Model 2 predicts that there are two fundamentally different “infectious nonself”/“danger” reactions of the innate immune system that direct T cell maturation along either the type-1 or type-2 pathway. Whereas both induce the up-regulation of costimulatory molecules on APC, one type of stimulus would induce IL-12 production, e.g., in dendritic cells (67), while the other would stimulate IL-4 and/or IL-6 production, e.g., in mast cells or eosinophils (68), the latter creating a cytokine microenvironment that favors type-2 T cell differentiation (69–71). The oils that IFA consists of might directly induce IL-4 or IL-6 production or might cause a sterile inflammation associated with prevalently type-2 cytokine microenvironment.

Model 3 predicts that there are two thresholds in the magnitude of the “infectious nonself” or “danger” reaction that define whether an Ag encounter results in tolerance, in a type-2 response, or in type-1 immunity. The recognition of Ag on resting APC by T cells, which would be below the first threshold (no or minimal “danger”), results in clonal inactivation and deletion in the absence of the induction of the “second signal,” as originally postulated and as it seems to occur after i.v. injection of deaggregated protein (72) and during naturally developing self-tolerance. Beyond a certain level of stimulation of the innate immune system, between the first and second thresholds, the “second signal” becomes engaged on APC, but IL-12 is not induced: moderate “danger” results in type-2 immunity. Once the magnitude of stimulation of the innate immune system exceeds the second threshold, the production of IL-12 is also induced: great “danger” causes type-1 immunity. In this model gradations of the same inflammatory reactions of the innate immune system therefore determine the type-1 or type-2 environment for T cell differentiation. In a modification of this model, it is conceivable that the strength of signal 1 and signal 2 synergistically define the class of the response (strong 1st × weak 2nd = weak 1st × strong 2nd). Our finding that OVA peptide 323–339 induced a mixed response when injected in IFA can be interpreted along these lines.

While it remains unclear why IFA acts as a strong type-2 adjuvant, our data provide clues about what it takes to generate a type-1 adjuvant: the presence of microbial products that signal “infectious nonself.” Confirming this notion, we found that supplementing IFA with pertussis toxin, LPS, or with oligonucleotides containing the CpG motif characteristic of bacterial DNA (substances that induce IL-12 production) all yield adjuvants that show the type-1-polarizing properties of CFA (Ref. 73, and T.F., unpublished observations). This information might be of particular relevance for human adjuvant design because CFA is not suited to human use due to the induction of severe granulomatous reactions and because aluminum, the only adjuvant recommended for human use, has type-2-inducing properties (Figs. 3 and 4).

We wish to point out that the polarizing effects of adjuvants reported here apply only to the priming of naive T cells. In ongoing experiments, we find that the response induced by the primary

CFA or IFA immunization largely maintains its type-1 or type-2 cytokine recall and Ab isotype profile after the mice are reinjected with the opposite type of adjuvant (data not shown). This finding is consistent with the notion that, unlike naive T cells, memory cells are committed to their type-1 or type-2 lineages (74). The data suggest that even after a primary injection adjuvants will not polarize a response toward the type-1 or type-2 pathway if the Ag-specific T cells are not naive but have been preprimed to type-1 or type-2 memory cells by cross-reactive environmental stimulation or by homologous self-proteins (22, 75). Furthermore, we found that IFA/Ag preparations needed to be injected i.p. (Fig. 3) and to be free of contaminating LPS or CpG-containing-DNA to induce pure type-2 responses, otherwise the IFA-induced response will be switched to type-1 (73). The same applies if the mouse colony is infected.

As with mice of different genetic backgrounds, humans also show considerable heterogeneity in their type-1/type-2 bias (76); this is a factor that must be overridden by vaccination protocols to ensure uniform success. Once type-1/type-2-polarizing adjuvants suitable for human use have been identified, exploiting the intrinsic properties of these adjuvants should be the simplest, cheapest, and most reliable subunit vaccination strategy for selectively engaging the desired class of immune response.

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