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Analysis of Immunization Route-Related Variation in the Immune Response to Heat-Killed *Salmonella typhimurium* in Mice

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In examinations of the factors regulating the quality and quantity of the immune response to *Salmonella typhimurium*, we have shown previously that live and heat-killed preparations of *S. typhimurium* can induce gamma interferon-dominant and interleukin-4-dominant immune responses, respectively, upon intraperitoneal (i.p.) immunization of BALB/c mice. Using this system to investigate the role of the route of immunization in the immune response, we show in the present study that i.p. immunization with heat-killed *S. typhimurium* generates a quantitatively better immune response than does intradermal (i.d.) immunization. The quantitative differences observed between the i.p. and i.d. routes are apparent in the amount of *S. typhimurium*-specific antibodies produced, the extent of responses in T-cell proliferation assays, and the quantities of lymphokines generated. However, the ratios of immunoglobulin (Ig) isotypes [IgG1/IgG2a] are comparable and the relative dominance of interleukin-4 over gamma interferon is seen in both i.p.- and i.d.-immunized mice, suggesting that the predominant T-cell effector pathways triggered are not qualitatively dependent on the route of immunization. An examination of the antigenic profile recognised by the B-cell and T-cell responses in i.p.- versus i.d.-immunized mice shows that while the Western immunoblot patterns recognized by serum antibodies from the two groups of mice were not significantly different, T cells from i.p.-immunized mice recognized a broader spectrum of antigens in an immunoblot assay than did those from i.d.-immunized mice. These data suggest that there may be a significant difference in the antigen-processing ability of peritoneal and dermal antigen-presenting cells for complex antigenic formulations such as bacterial vaccines.

The protection conferred by the immune system against infections depends on both the amount and the type of immune response generated. Therefore, the control of both these parameters is a matter of concern in choosing vaccination strategies. In clinical situations, a major factor in vaccination strategies is the route of immunization. The effects of various routes of immunization on the level and type of the immune response are therefore of interest. Immunization with a single protein, ovalbumin, by different routes (intraperitoneal [i.p.] versus intranasal) has been shown to result in the generation of qualitatively different immune response patterns (4). The intradermal (i.d.) route of immunization has been a route of clinical choice for obvious reasons of convenience and has been shown to be more effective than other parenteral routes in some cases. When complex bacterial antigens such as *Salmonella enteritidis* and *Mycobacterium* species are used, the i.d. route is better able to generate a delayed-type hypersensitivity (DTH) response than the i.p. route (3, 15).

CD4 T cells are critical to the generation of an effective immune response to exogenous antigens (8). The two functional categories of CD4 T cells, Th1 and Th2, appear to be responsible for the maturation of different effector immune mechanisms. Th1 CD4 T cells preferentially make gamma interferon (IFN-γ), which activates macrophages to kill intracellular facultative parasites (13). Such pathogens cannot be eliminated completely by antibodies alone. The Th2 CD4 T cells, on the other hand, preferentially make interleukin-4 (IL-4), which is central to the maturation of antibody responses (13) and would thus aid in the clearance of extracellular pathogens from the body. Control of the type of immune response generated is therefore of major importance in designing vaccines. Work on cutaneous (14) and visceral (5) leishmaniasis, schistosomiasis (7), and candidiasis (11) has demonstrated that the predominant Th subset in the immune response can vary with the type of organism and strain of mouse as well as with the route of exposure to the immunogen (13).

Salmonella typhimurium is considered a candidate vector in vaccine design (1, 9, 12). We have been studying the development of immune responses following immunization with *S. typhimurium* by using different formulations of the organism and different routes of immunization in a mouse model. We reported previously that when the i.p. route of immunization is used, sublethal doses of live *S. typhimurium* give rise to an IFN-γ-dominant, Th1-like immune response whereas heat-killed organisms generate an IL-4-dominated Th2-like response (17). We now report that immunization with heat-killed organisms via different routes of immunization leads to major quantitative differences in the resultant immune response. There is also a significant difference in the antigenic repertoires recognized by immune T cells from i.p.- versus i.d.-immunized mice, suggesting that dermal antigen-presenting cells (APCs) may be limited in their ability to process complex mixtures of particulate immunogens and that this may have measurable consequences on the immune response generated.

MATERIALS AND METHODS

Bacteria. The strain of *S. typhimurium* used was a clinical isolate from Lady Harding Medical School, New Delhi, India. Bacterial stocks were stored as aliquots in glycerol broth at −70°C after biochemical and serological confirmation of identification. Bacteria were grown to log phase in Luria broth, washed in phosphate-buffered saline (PBS; pH 7.2) and killed in a boiling-water bath for 45 min. Completion of killing was confirmed by absence of viable bacteria after treatment. A soluble extract of *S. typhimurium* was prepared by sonicating *S. typhimurium* in a Sonifier (Branson, Danbury, Conn.) containing PBS with 10 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.) as a

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protease inhibitor and centrifuged at 100,000 × *g* for 60 min at 4°C. This sonicate was used in the assays of immune responses.

Mice. BALB/c mice (Jackson Laboratories, Bar Harbor, Maine) bred in the Small Animal Facility of the National Institute of Immunology, New Delhi, India, were used for all experiments. Six to ten mice were used per group for immunizations. The mice were bled under inhalation anesthesia from the retro-orbital venous plexus, and sera were separated for antibody measurements.

Immunization. Optimal doses for immunization were determined by preliminary titration (data not shown). All bacteria, live or killed, used for immunizations were washed and resuspended in PBS for injection. Two doses of 10^8 heat-killed *S. typhimurium* organisms were given 1 week apart by the i.p. or i.d. route. For live *S. typhimurium* immunization, the first dose was 10^3 organisms and was followed 1 week later by 10^4 organisms. The i.d. immunizations were given on the insides of both thighs.

Assay of total *S. typhimurium*-specific antibody. Polyvinyl chloride microtiter plates (Falcon) were coated with *S. typhimurium* sonicate (10 μg/ml), and *S. typhimurium*-specific antibodies in the immune sera were detected with a goat anti-mouse immunoglobulin (Ig)-peroxidase conjugate; *o*-phenylenediamine tetrahydrochloride (Sigma) and H_2O_2 were used as the revealing reagents. Concentrations were calculated on the basis of a standard curve of affinity-purified mouse Ig run in a parallel enzyme immunoassay.

IgG subclass analysis. Polyclonal anti-IgG1 and anti-IgG2a (Sigma) were used to determine titers of *S. typhimurium*-specific IgG subclasses in each serum sample by enzyme immunoassay. Titers were determined, from an extensive titration of each serum sample, as the dilution of serum required to reach half-maximal absorbance and were expressed as reciprocal log values. Monoclonal antibodies of each subclass were used to confirm the specificity of the assays (data not shown).

DTH response. *S. typhimurium* sonicate was injected in one hind footpad (50 μg in 50 μl of PBS); the other footpad received PBS as a control. The increase in footpad thickness was measured with a spring-loaded measuring caliper (Mitutoyo) 24 and 48 h after injection. The DTH response is expressed as the difference (in millimeters) between the thickness of the test and control footpads at 48 h.

T-cell proliferation assays. Triplicate cultures of responder splenic or inguinal lymph node cells (3 × 10^5 cells per well) were incubated with graded doses of *S. typhimurium* sonicate in 200 μl of Dulbecco's minimal essential medium (Gibco, Grand Island, N.Y.) containing 10% fetal calf serum (Hyclone, Logan, Utah), 10 mM glutamine (Sigma), 50 μM β-mercaptoethanol (Sigma), and antibiotics in flat-bottom 96-well plates. Supernatants were collected after 60 h for lymphokine assays, and the plates were pulsed with 1 μCi of [^{3}H]thymidine (Amersham, Amersham, United Kingdom) for 8 to 16 h in a 96- to 110-h assay. The plates were harvested onto fiberglass filters and counted by liquid scintillation spectroscopy (Betaplate; LKB-Pharmacia, Uppsala, Sweden). Background counts were uniformly <1,000 cpm.

Lymphokine bioassays. The culture supernatants harvested at 60 h during the T-cell proliferation assays were used for all lymphokine estimations. The CTLL-2 cell line (TIB 214; American Type Culture Collection [ATCC], Rockville, Md.) was used to measure IL-2 and IL-4 levels. The contribution of IL-2 and IL-4 to the proliferation of CTLL-2 was assayed with the anti-IL-4 monoclonal antibody 11B11 (HB188; ATCC). Concentrations of the lymphokines present were calculated from standard curves of recombinant IL-2 and IL-4 (Genzyme, Cambridge, Mass.). The anti-IL-4 used was sufficient to inhibit the proliferation induced by 100 U of recombinant IL-4 per ml without affecting IL-2-induced responses (17). For estimation of IFN-γ, the inhibition of growth of WEHI279 cells (CRL1704; ATCC) was used as a bioassay. To confirm the inhibitory effect of IFN-γ, an anti-IFN-γ monoclonal antibody (R4-6A2, HB170; ATCC) was used. Recombinant IFN-γ (Boehringer, Mannheim, Germany) was used as a standard to calculate the concentration of IFN-γ in the culture supernatants.

T-cell immunoblot analysis. The proteins in *S. typhimurium* sonicate were separated by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions with 12% acrylamide on a minigel apparatus (Bio-Rad, Richmond, Calif.). Separated proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) by electroblotting in Tris-glycine buffer containing 20% methanol. Protein transfer was confirmed by staining with 0.01% amido black (Sigma). The membranes were prepared for proliferation assays as described previously (19). In brief, membranes were cut horizontally into strips, dissolved in dimethyl sulfoxide (Sigma) for 1 h, and precipitated by addition of carbonate-bicarbonate buffer (pH 9.2) with intermittent mixing. The dimethyl sulfoxide was removed by extensive washing with PBS and this precipitate resuspended in Dulbecco's minimal essential medium. Graded doses of this particulate antigenic preparation were used in T-cell proliferative assays as mentioned above.

Western immunoblot analysis. The proteins in *S. typhimurium* sonicate were separated and transferred to nitrocellulose membrane as mentioned above. Antigens recognized by immune sera were detected with appropriate dilutions of these sera. Goat anti-mouse Ig-peroxidase conjugate was used to detect the bound Ig, with 4-chloro-1-naphthol (Sigma) and H_2O_2 as revealing agents.

Statistical analyses. All statistical analyses were carried out with Student's *t* test and are shown wherever appropriate.

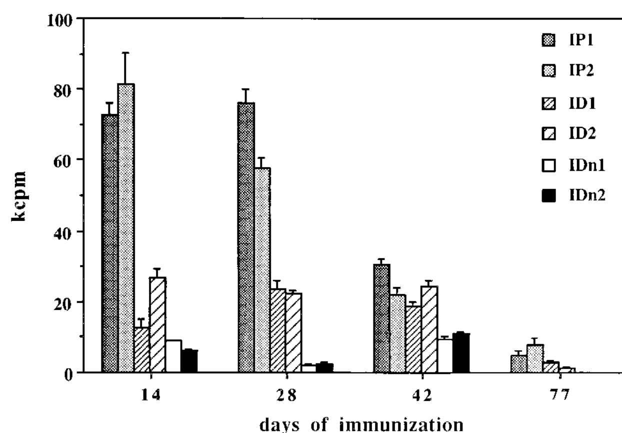

FIG. 1. Proliferative responses of cells from individual i.p.- and i.d.-immunized mice on various days after immunization with 3 μg of *S. typhimurium* sonicate per ml. IP1 and IP2 refer to results from spleen cells of mice immunized i.p., while ID1 and ID2 refer to the results from spleen cells of mice immunized i.d. IDn1 and IDn2 refer to the results from draining inguinal lymph node cells from i.d.-immunized mice. Each bar represents the response of a single mouse at the stated time point and is shown as mean ± standard error for triplicate cultures. kcpm, thousands of counts per minute.

RESULTS

The magnitude of the immune response is higher in i.p.-immunized than in i.d.-immunized mice. *S. typhimurium*-specific antibody levels in serum were 30-fold higher in i.p.-immunized mice at day 28 (13.0 ± 2.7 μg/ml after i.p. immunization versus 0.44 ± 0.09 μg/ml after i.d. immunization [$P < 0.001$]). These levels were higher (by about 10-fold) in i.p.-immunized mice earlier (2 weeks) as well as later (10 weeks) (data not shown).

There was also a significant difference in the T-cell proliferative response observed in vitro. Figure 1 shows that immunization by the i.p. route generated an excellent in vitro T-cell proliferative response which declined with time, whereas i.d. immunization generated a comparatively poor response at all time points tested in both regional lymph nodes and spleen cells. For convenience, the results of responder cells from individual mice for a dose of 3 μg of *S. typhimurium* sonicate per ml are shown in Fig. 1, but the difference is seen at all doses of antigen tested over a 30-fold range and is representative of at least three such experiments.

Culture supernatants collected at 60 h from the T-cell proliferation assays done on day 28 of immunization were analyzed for their IL-2 and IL-4 content by bioassays. Figure 2 shows that significantly higher levels of IL-4 were detectable in cultures from i.p.-immunized mice than in cultures from i.d.-immunized mice (Fig. 2A). The levels of IL-2 were low but comparable (Fig. 2B) for the two routes of immunisation. These differences are also representative of three different experiments.

These three findings together suggest that the magnitude of the immune response generated by identical amounts of *S. typhimurium* is much lower after i.d. than i.p. immunization.

The dominance of a Th2-like response is seen in both i.p.- and i.d.-immunized mice. Despite the quantitative differences in antibody production, the ratio of IgG1 to IgG2a is comparable in i.p.- and i.d.-immunized mice and significantly lower in mice immunized with live *S. typhimurium* (Fig. 3) (17), suggesting that similar subclass-specific antibody isotype-switching mechanisms are operative in both routes of immunization.

DTH was also estimated at various time points (from 2 to 12 weeks) after immunization by injection of *S. typhimurium* son-

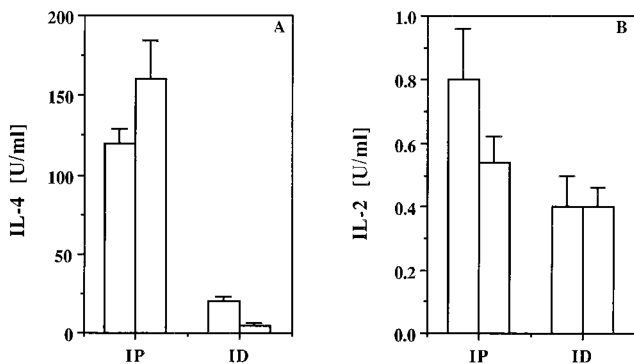

FIG. 2. Estimation of IL-4 (A) and IL-2 (B) in culture supernatants of responder spleen cells stimulated with 3 μg of *S. typhimurium* sonicate per ml. Each bar represents lymphokine levels from an individual mouse and is shown as the mean and standard error of triplicate cultures.

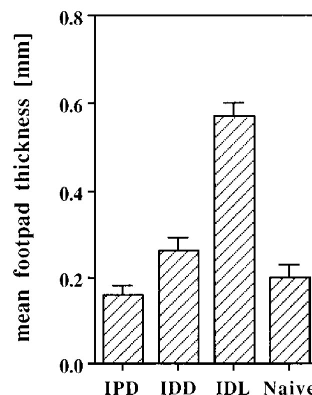

FIG. 4. DTH response in mice immunized with killed *S. typhimurium* i.p. (IPD) or i.d. (IDD) compared with mice immunized with live *S. typhimurium* i.d. (IDL) or nonimmunized (Naive). IPD and IDD groups did not mount a significant DTH response ($P > 0.05$ for IDD, and $P > 0.1$ for IPD) over the naive group, whereas the positive control IDL group showed a very significant DTH ($P < 0.001$). Data are from six mice per group on day 28 of immunization.

icate into the footpads. As shown in Fig. 4, both i.p. and i.d. immunization generated a poor DTH response compared with immunization with live *S. typhimurium*, which was used as the positive control. There was no significant increase in footpad thickness as a result of DTH on day 28 of immunization either in the i.p.- or in the i.d.-immunized mice over the background value observed in nonimmunized mice. At no time point, in fact, do mice immunized with heat-killed *S. typhimurium* show any significant DTH over nonimmunized naive mice (data not shown).

Culture supernatants collected at 60 h from the T-cell proliferation assays done on day 28 of immunization were analyzed for their IFN-γ content by bioassay. The amount of IFN-γ present in these culture supernatants was <1 U/ml (the detection limit of our assay system), in contrast to that present in supernatants from a control group of mice immunized i.d. with live *S. typhimurium*, which was 26 U/ml.

These data suggest that the relative dominance of a Th2-like response, which we previously demonstrated for i.p. immunization with killed *S. typhimurium* (17), is also seen in mice immunized by the i.d. route.

Analysis of the antigenic repertoires *S. typhimurium* recognized by B- and T-cell responses in i.p.- and i.d.-immunized mice. Since the levels of *S. typhimurium*-specific antibodies are higher in i.p.-immunized mice, it was of interest to examine whether the difference is due to the recognition of fewer antigens from *S. typhimurium* by the i.d.-immunized mouse sera. Figure 5 shows a Western blot analysis which demonstrated that no major differences were apparent in the antigenic profiles recognised by pooled serum antibodies from the two groups of mice. Minor differences (such as that around 27.5 kDa in the experiment shown in Fig. 5) have not been found to be reproducible, and the broad patterns vary little between experiments or even individual mice. The antigenic specificities recognized in both modes of immunization are spread over a broad molecular mass range, as expected in a polyclonal response to an antigenic source as complex as *S. typhimurium*. Results shown in Fig. 5 are for day 28 of immunization and are representative of four experiments and two time points.

In contrast to the antibody responses, the antigenic profiles recognized by T cells are significantly different between the two modes of immunization. Since the proliferative responses of spleen cells are better than those of the draining lymph node cells in i.d.-immunized mice, spleen cells were used from both i.p.- and i.d.-immunized mice in a T-cell immunoblot assay. As seen in Fig. 6, the responses of spleen cells from i.p.- and

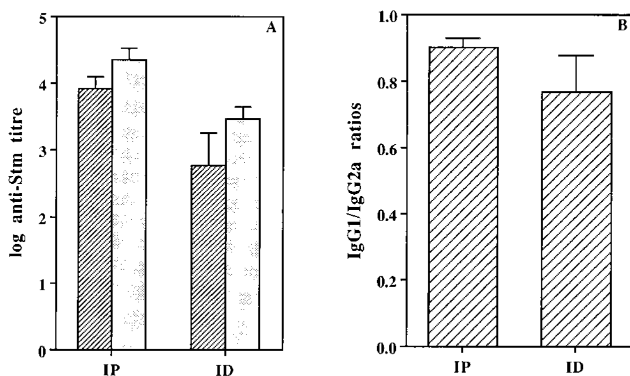

FIG. 3. The ratio of IgG1 to IgG2a is comparable ($P > 0.2$) in spite of the differences in anti-*S. typhimurium* antibody titers for both subclasses ($P < 0.05$ for IgG1 and $P < 0.01$ for IgG2a) in the two groups on day 28 of immunization. (A) Log titers for IgG1 (hatched bars) and IgG2a (stippled bars) for i.p.- and i.d.-immunized mice (mean ± standard error for eight mice per group). (B) Ratio of IgG1 to IgG2a for the same groups. These results are representative of three experiments.

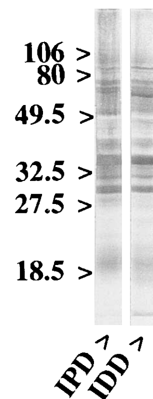

FIG. 5. Western blot analysis on pooled immune sera from i.p.- (IPD) and i.d.- (IDD) immunized mice on day 28 of immunization. Most of the bands picked up by the antibodies from the *S. typhimurium* sonicate are shared between the two groups. Numbers at left indicate molecular mass in kilodaltons.

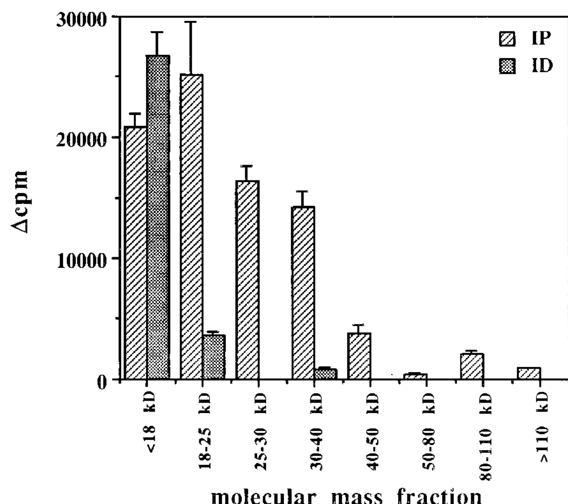

FIG. 6. T-cell immunoblot assay showing proliferative responses to various molecular mass fractions (as indicated) from the *S. typhimurium* sonicate. Responses of splenic T cells from i.p.- and i.d.-immunized mice on day 42 of immunization are shown as means and standard errors of triplicate cultures. The background levels of thymidine incorporation in the presence of unblotted nitrocellulose granules were 5,724 ± 410 cpm for the cells from i.p.-immunized mice and 6,877 ± 1,258 cpm for the cells from i.d.-immunized mice. The data are representative of three independent experiments.

i.d.-immunized mice to the <18-kDa fraction are comparable, but responses to the subsequent higher-molecular-mass fractions are much better in cells from i.p.-immunized mice than in cells from i.d.-immunized mice. Thus, the proliferative response to different antigenic fractions of *S. typhimurium* sonicate was directed against a wide range of molecular mass fractions in i.p.-immunized mice, whereas it was restricted to a narrower range of fractions, mainly those below 18 kDa, in i.d.-immunized mice. The preponderance of response seen to the lower-molecular-mass fractions could be due to better transfer of proteins onto nitrocellulose, but that finding is common to T cells from both the i.p.-immunized and the i.d.-immunized mice, suggesting that for the same amount of protein transferred, T cells from i.p.-immunized mice recognize higher-molecular-mass fractions much better than do those from i.d.-immunized mice.

DISCUSSION

We have analyzed parameters of the immune response generated by immunization with *S. typhimurium* because of its importance as a candidate recombinant vaccine vector. Here we report that heat-killed *S. typhimurium* elicits a quantitatively superior immune response when administered by the i.p. route than by the i.d. route, as measured by *S. typhimurium*-specific antibody levels, T-cell proliferation, and cytokine secretion. The relative dominance of IL-4 also persists regardless of the route used. A major difference between the immune responses generated by the two routes appears to lie in the range of antigens recognized by responding T cells. T cells from i.d.-immunized mice recognize a relatively narrow range of antigens compared with those from i.p.-immunized mice, indicating that the route of immunization may significantly influence the antigenic repertoire recognized by immune T cells.

The *S. typhimurium*-specific antibody response in i.d.-immunized mice was lower than that in i.p.-immunized mice. However, the Western blot analysis suggested that there are no significant differences in the repertoire of *S. typhimurium* antigens recognized at the B-cell level after immunization by the different routes. Serum antibodies from both groups of mice recognized a wide array of antigens spanning the entire molecular mass range. The differences observed in the total antibody levels in serum despite similar antigenic profiles may thus be due to differences in the amount of help provided to *S. typhimurium*-specific B cells in vivo between the two modes of immunization. Since the immunogen is in particulate form, B cells reactive to many different available antigens on the bacterial particles would be able to take up the bacteria. They would then present a peptide from associated antigens (that they do not themselves recognize) even to a very restricted repertoire of T cells. Thus, B cells of a diverse antigenic repertoire could attract T-cell help even from a restricted set of T cells as in i.d.-immunized mice. The magnitude of the total T-cell response would then affect the total quantity of the antibody produced rather than its antigen-specific distribution. Also, we have not detected significant differences between *S. typhimurium*-specific IgM levels in the two groups in preliminary experiments (data not shown).

Differential effects of various cytokines on the antibody isotype-switching patterns have been demonstrated previously (16). IL-4 induces a preferential switch from IgM to IgG1 (and IgE), whereas IFN-γ has been shown to facilitate a switch from IgM to IgG2a (16). Whether it is the relative ratio of the two lymphokines which determines the relative proportions of IgG1 and IgG2a antibodies or whether it is the earlier appearance of one lymphokine than the other is not clear either. In our system, we find very low to undetectable levels of IFN-γ (<1 U/ml) in both groups but comparatively higher levels of IL-4 in i.p.-immunized mice. Despite that, the ratios of IgG1 to IgG2a are comparable for the two modes (Fig. 3B), suggesting that levels of IL-4 beyond a certain threshold of dominance over IFN-γ may not affect the isotype profile significantly. In the absence of detectable IFN-γ, significant levels of IgG2a are present in the immune sera, and IFN-γ-independent mechanisms may be responsible for the switching observed (2).

The absence of detectable IFN-γ in the T-cell stimulation assays correlates well with the DTH results. We (17) and others (18) have shown that DTH is normally associated with significant levels of IFN-γ. It was shown previously that i.d. immunization with killed bacterial preparations such as *S. enteritidis* or rapidly growing *Mycobacterium* species such as *Mycobacterium vaccae* results in the induction of a good DTH response, whereas i.p. immunization does not (3, 15). For slow-growing mycobacteria, both these routes are equally competent for the induction of DTH by killed organisms (15). These previous data suggest that commitment to the Th1- versus the Th2-like pathway may be modified depending on the route of immunization. However, our data with *S. typhimurium* do not show such a modulation of the type of immune response generated, since the Th2-like-dominant phenotype of the immune response generated by i.p. immunization with killed *S. typhimurium* (17) appears to remain unchanged in i.d.-immunized mice. In fact, the Th1-like dominance of the response that we previously demonstrated with i.p. injection of live *S. typhimurium* is also seen with i.d. injection (Fig. 4, IFN-γ levels, and data not shown).

The major difference in the immune responses to killed *S. typhimurium* administered via either the i.p. or the i.d. route thus appears to be quantitative rather than qualitative. Our data on recognition of nitrocellulose-blotted *S. typhimurium* antigens by immune T cells (Fig. 6) indicate a possible explanation for this quantitative difference. There was a marked difference in the range of antigens recognized by T cells after

i.p. versus i.d. immunization. Within the limitations of the technique, it is evident that T cells from i.d.-immunized mice recognize a relatively narrow range of antigenic fractions located in the low-molecular-mass regions (<18 kDa). The T cells from i.p.-immunized mice also recognize the low-molecular-mass fractions very well, but they also recognize other fractions to a very significant extent. The high responses to the low-molecular-mass fractions in both groups of mice may be due at least partly to the near-complete transfer of proteins taking place in the low-molecular-mass range. The fact that cells from i.d.-immunized mice respond at least as well to the <18-kDa fraction suggests that their failure to respond well to most other fractions is not a consequence of the overall lower anti-*S. typhimurium* T-cell reactivity in i.d.-immunized mice. Thus, if the responses to all other fractions are compared with the responses to the <18-kDa fraction, it is evident that, for the same amount of protein transferred onto nitrocellulose, T cells from i.p.-immunized mice recognize higher-molecular-mass fractions to a far greater degree than do those from i.d.-immunized mice. This cannot be due to any difference in the antigen-processing capabilities and/or accessory signaling potential of APCs present in the in vitro recall assay, since both these assays were done with splenic cells. This clearly suggests that the difference in the repertoire of antigens recognized by T cells from i.p.-immunized versus i.d.-immunized mice is a consequence of factors associated with priming events rather than the recall response.

The two routes of immunization used here potentially expose the immunogen (*S. typhimurium* bacterial suspension in this case) to different sets of APCs for the primary encounter. When the i.p. route is used, peritoneal macrophages will be the major population of APCs available. When the i.d. route is used, Langerhans cells would be the main APCs involved. Dendritic cells are considered to be the most important cells in the priming process (6), and Langerhans cells belong to the dendritic cell lineage. However, dendritic cells (including Langerhans cells) appear to be inefficient in phagocytosis compared with cells of the macrophage lineage. Since we are using a particulate-antigen formulation for immunization, uptake by phagocytic macrophages may be an important factor affecting the availability of various antigenic proteins from *S. typhimurium* for presentation to T cells, and this may be the reason enabling T cells from i.p.-immunized mice to recognize a larger repertoire of *S. typhimurium* antigens than do T cells from i.d.-immunized mice.

It is noteworthy that Langerhans cells have a higher level of cell surface major histocompatibility complex class II molecules than do many other cell types (10). Despite this, the immune response to killed *S. typhimurium* appears to be weaker in i.d.-immunized than i.p.-immunized mice. This may suggest that antigen internalization plays a crucial role in immune responses to particulate antigens. Differences in ligand density have been shown to lead to qualitative shifts in the immune response generated (8). Whether the differences in ligand densities between the i.p. and i.d. modes of immunization lead in some cases (like our data) to a quantitative difference in the immune response and in other cases to a qualitative change, as reported for *S. enteritidis* or *M. vaccae* (3, 15), remains to be examined.

ACKNOWLEDGMENTS

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