INFECTION AND IMMUNITY, May 1994, p. 1881–1888
0019-9567/94/$04.00+0

Vol. 62, No. 5

Antigen-Specific T-Cell Responses during Primary and Secondary *Listeria monocytogenes* Infection

SABINE DAUGELAT, CHRISTOPH H. LADEL, BERND SCHOEL, AND STEFAN H. E. KAUFMANN*

Department of Immunology, University of Ulm, Albert-Einstein-Allee 11, D-89070 Ulm, Germany

Received 22 October 1993/Returned for modification 19 November 1993/Accepted 28 January 1994

Although murine listeriosis is a widely used experimental model for the analysis of cell-mediated immunity, there is little information about individual T-cell antigens of *Listeria monocytogenes* which are recognized during primary and secondary infection. To study the antigen responses of *L. monocytogenes*-reactive T cells, somatic and secreted listerial proteins were separated by two-dimensional gel electrophoresis and subsequently divided into 480 liquid fractions. Antigen-specific T cells isolated from mice at different times of primary and secondary listeriosis were tested for their capacity to proliferate with distinct protein fractions. Supernatants of these cultures were screened for the production of gamma interferon, interleukin-4 (IL-4), and IL-10. Proliferation of antigen-specific T cells correlated with the production of high concentrations of gamma interferon, whereas IL-4 and IL-10 production in response to listerial protein fractions could not be detected. During both primary and secondary listeriosis, T cells recognized a multitude of somatic and secreted proteins rather than one or a few dominant antigens. Secreted proteins were recognized before somatic proteins, and T cells recognized different fractions in secreted and somatic proteins.

Murine listeriosis caused by the facultative intracellular bacterium *Listeria monocytogenes* is widely used as a model system for infectious diseases involving cell-mediated immunity (12, 16, 23). The outcome of an infection with *L. monocytogenes* is determined by numerous parameters, including inoculum size, bacterial virulence, and susceptibility of mouse strains (19). Infection of genetically resistant C57BL/6 mice with a sublethal dose of *L. monocytogenes* results in acute infection. The number of bacteria reaches a maximum in the spleen and liver at about day 3 to 4 postinfection (p.i.) and then decreases rapidly, and the bacteria are eliminated by day 7 to 10 p.i.

Primary listeriosis comprises two phases. First, natural or innate resistance develops, which involves natural killer cells, neutrophils, and macrophages. This phase is succeeded by a specific immune response, which is mediated by antigen-specific T lymphocytes and effected by macrophages. A complex network of cytokines, including gamma interferon (IFN-γ), interleukin-1 (IL-1), IL-2, IL-4, IL-6, IL-10, IL-12, and tumor necrosis factor alpha, are involved in early antibacterial defense (reviewed in references 1, 22, and 32). Antigen-specific T cells can be detected from day 3 to 4 p.i. on, and $CD4^+$, $CD8^+$, and γδ T cells contribute to clearance of bacteria (15, 16, 25, 27). After the acute infection has resolved, mice remain immune for a long time. Thus, challenge with an otherwise lethal inoculum results in rapid elimination of bacteria in immune mice. This heightened response against secondary listeriosis depends on antigen-specific memory T cells, as has been shown by adoptive transfer and in vivo depletion experiments (24, 26, 28).

L. monocytogenes-reactive T cells recognize bacterial peptides presented by products of the major histocompatibility complex (MHC) on the surface of antigen-presenting cells. Bacteria consist of several thousand proteins, all of which represent potential antigens. Although some of these proteins have been claimed to be major T-cell antigens, virtually nothing is known about the overall antigen repertoire of T cells during primary and secondary infection. In particular, it remains unclear whether the T-cell response is (i) focused on a restricted number of dominant antigens or (ii) directed towards a large range of antigens. Moreover, it is unknown whether the T-cell response is biased towards (i) secreted antigens from bacteria surviving in host cells or (ii) somatic antigens from killed microbes. We wanted to study this question by using *L. monocytogenes* proteins separated by two-dimensional gel electrophoresis (native conditions used in the second dimension [11]) and specific T cells isolated from mice at different stages of infection. These cells were tested for their capacity to proliferate in response to fractionated somatic and secreted proteins of *L. monocytogenes* and for cytokine secretion to gain insight into their possible in vivo function. We demonstrate that during primary and secondary listeriosis, a multitude of protein fractions are recognized by antigen-specific T cells, that proliferation is accompanied by the production of high concentrations of IFN-γ, and that secreted proteins are recognized before somatic proteins.

MATERIALS AND METHODS

Bacteria and bacterial antigens. *L. monocytogenes* EGD, obtained from the homogenized spleen of an infected mouse, was grown in tryptic soy broth (Difco, Detroit, Mich.) to the mid-logarithmic phase. Aliquots were stored at −70°C. The number of viable bacteria was determined by plating serial 10-fold dilutions on tryptic soy agar (Difco). For infection, an aliquot was thawed, diluted in phosphate-buffered saline (PBS) as required, and injected in a volume of 0.2 ml. For production of bacterial lysates, *L. monocytogenes* organisms were grown in tryptic soy broth overnight, centrifuged, washed once with PBS, and killed in 70% ethanol for 90 min. Bacteria were washed twice and resuspended in a small volume of 20 mM Tris-HCl buffer (pH 7.5) containing 5 mM $MgCl_2$. An equal weight of glass beads (0.18-mm diameter; B. Braun Melsungen, Melsungen, Germany) and a cocktail of protease inhibitors (1 μM pepstatin, 1 μM leupeptin, and 200 μM phenylmethylsul-

* Corresponding author. Mailing address: Department of Immunology, University of Ulm, D-89070 Ulm, Germany. Phone: 0731/502-3361. Fax: 0731/502-3367. E-mail: Kaufma@dulruu51.bitnet.

fonyl fluoride; Boehringer, Mannheim, Germany) were added, and the bacteria were disrupted in a glass bead mill (Braun). Crude lysates were treated with DNase and RNase (Sigma, St. Louis, Mo.) and centrifuged at 107,000 × *g* for 30 min. The protein concentrations of the supernatants were determined by the bicinchoninic acid protein test (Pierce, Rockford, Ill.). Aliquots were stored at −20°C.

For production of listerial culture filtrate proteins, tryptic soy broth was filtered through an Amicon YM-10 membrane (Amicon, Beverly, Mass.), and the protein-free eluate was sterilized by autoclaving. *L. monocytogenes* was grown in this modified medium for 18 h because the growth rate was lower than in unmodified tryptic soy broth. The bacteria were sedimented, and the culture filtrate was sterile filtered. Proteins of >10 kDa were concentrated by ultrafiltration, washed with distilled water to remove salts, and tested for protein concentration with the Bio-Rad protein assay (Bio-Rad, Richmond, Calif.). Aliquots were stored at −20°C. Purified p60 was a kind gift of W. Goebel, Institute of Microbiology, University of Würzburg, Germany. Listeriolysin was purified from concentrated culture filtrates of *L. monocytogenes* by ammonium sulfate precipitation, hydrophobic interaction chromatography, and finally cation-exchange chromatography. All fractions were screened by a hemolytic assay (36).

Mice. Male C57BL/6 mice, 8 to 12 weeks old, were bred and maintained under specific-pathogen-free conditions at the University of Ulm and used in all experiments. For primary infection, 5×10^3 to 10×10^3 *L. monocytogenes* organisms were injected intravenously into the tail vein. For secondary infection, 1×10^5 to 2×10^5 bacteria (a lethal dose for naive mice) were injected 14 weeks after the first infection by the same route. The number of organisms in individual inocula was confirmed by plating an aliquot of the bacterial suspension on tryptic soy agar plates. Mice were killed by cervical dislocation, and their spleens were removed aseptically.

Two-dimensional PAGE. For polyacrylamide gel electrophoresis (PAGE) in the first dimension, proteins were focused according to their isoelectric points (pIs) as described previously (11), with slight modifications. In short, denaturing gels containing 8 M urea (Bio-Rad) were run in glass tubes (3.5 mm inside diameter) for 6,000 V · h. A mixture of ampholytes (40% 4-6, 40% 5-7, 20% 3-10; Serva, Heidelberg, Germany) was used at 10% (vol/vol) to form a gradient from pH 7 to 4. Then, 100 μg of proteins was loaded on each gel. After equilibration in 60 mM Tris-HCl (pH 8.8) containing 10% glycerol for 45 min, gels were stored at −20°C. For the second dimension, linear 7.5 to 10% native polyacrylamide gradient gels were used. The final gel size (8.5 by 11 cm) fitted exactly the master plate of the Blotelutor with its grid of 24 by 20 fractions. Molecular weight standards were not used because they do not migrate according to their size in the native system.

Protein transfer. An electroelution device (Blotelutor; Biometra, Göttingen, Germany) was used to transfer 480 distinct liquid protein fractions from a two-dimensional gel as described earlier (11). Fractions were stored at −20°C. Before use, each fraction was diluted with 130 μl of plain Iscove's modified Dulbecco's medium (IMDM; Biochrom, Berlin, Germany), and duplicate samples of 20 μl were transferred into 96-well tissue culture plates (Nunc, Roskilde, Denmark) and sterilized with UV light.

T-cell proliferation assay. Spleens from infected animals were passed through steel mesh sieves to obtain single-cell suspensions. Erythrocytes were lysed with 0.17 M NH_4Cl, and the remaining spleen cells were washed three times in medium. Depletion of macrophages was performed in two steps. First, plastic-adherent cells were enriched by incubating spleen cells in an 600-ml tissue culture flask (Nunc) for 90 min at 37°C in 20 ml of IMDM supplemented with 10% heat-inactivated fetal calf serum (FCS; Boehringer), 100 U of penicillin-streptomycin (GIBCO, Paisley, Great Britain) per ml, 2 mM glutamine (GIBCO), 5×10^{-5} M 2-mercaptoethanol (GIBCO), and 1 μg of indomethacin (Sigma) per ml (referred to hereafter as complete medium). Nonadherent cells were carefully washed off with warm Hanks' balanced salt solution (HBSS; Biochrom) supplemented with 10% FCS (HBSS-FCS).

For the second depletion step, Sephadex G10 columns were prepared as follows. Five grams of Sephadex G10 (Pharmacia LKB, Uppsala, Sweden) was autoclaved in 20 ml of 0.85% NaCl and added to a 20-ml syringe plugged with silanized glass wool (Serva). Columns were rinsed with HBSS-FCS, and the nonadherent cells were loaded in a volume of 1 to 2 ml of HBSS-FCS. After 20 min of incubation at room temperature, cells were eluted with warm HBSS-FCS and used directly as a source of T cells at 0.7×10^5 to 1×10^5 cells per well. Fluorescence-activated cell sorting (FACS) analysis of these purified cell preparations showed that they comprised 30 to 45% T lymphocytes and 45 to 60% B lymphocytes. Since B cells do not contribute to proliferation and IFN-γ production in response to listerial antigens at the concentrations used here (17, 18), this cell population is referred to as T cells.

Syngeneic spleen cells irradiated with 3,000 rads were used as accessory cells at 2×10^5 to 3×10^5 per well. Although it is unlikely that infected macrophages were carried over from spleens, any remaining *L. monocytogenes* organisms would have been killed by the addition of antibiotics to the culture medium. Cells were cultured in complete medium in a total culture volume of 220 μl per well (200 μl of cells plus 20 μl of antigen). Controls contained unfractionated listerial lysate and listerial culture filtrate (titrated from 1 to 0.03 μg per well) and concanavalin A (ConA; Sigma; 0.5 to 0.12 μg per well) as a mitogen. Cultivation time was 2 days for ConA-stimulated cultures and 4 to 6 days for antigen fractions and controls. Supernatants (150 μl per well) were removed 6 h before termination of incubation. At this step, supernatants of duplicates were pooled and stored at −20°C. Then, 0.5 μCi of [^{3}H]thymidine (Amersham-Buchler, Braunschweig, Germany) was added to each well, and 6 h later the plates were frozen at −20°C. Cells were thawed and harvested onto glass fiber filtermats, and radioactivity was determined in a Betaplate counter (Skatron, Lier, Norway).

ELISA. Culture supernatants were screened for IFN-γ, IL-4, and IL-10 by double sandwich enzyme-linked immunosorbent assays (ELISAs) with two specific monoclonal antibodies recognizing different epitopes of the respective cytokines. Biotinylation of the second antibody allowed binding of streptavidin-conjugated alkaline phosphatase (Dianova, Hamburg, Germany) and detection with *p*-nitrophenylphosphate (Sigma). Murine recombinant IFN-γ (rIFN-γ), IL-4, or IL-10 was diluted in complete medium from 100 to 0.2 U/ml and used to obtain a standard curve. Reactions were stopped after 15 to 20 min with 0.5 M EDTA, pH 8.8, and the optical density was measured in an Intermed NJ-2000 Immunoreader (Nunc). The ELISA LITE software (Meddata Inc., New York, N.Y.) was used to calculate cytokine concentrations. Rat anti-murine IFN-γ hybridomas R4-6A2 (38) and AN18-17-24 (34) were kindly provided after subcloning by J. Langhorne, Max Planck Institute for Immunobiology, Freiburg, Germany (37); murine rIFN-γ was a generous gift of G. Adolf, Ernst-Boehringer-Institut für Arzneimittelforschung, Vienna, Austria. Rat anti-murine IL-4 hybridomas BVD4-1D11 and GVD6-24G2 as well as the rat-anti murine IL-10 hybridoma 2A5.7 were a generous gift of R. L. Coffman, DNAX Research Institute of Molecular

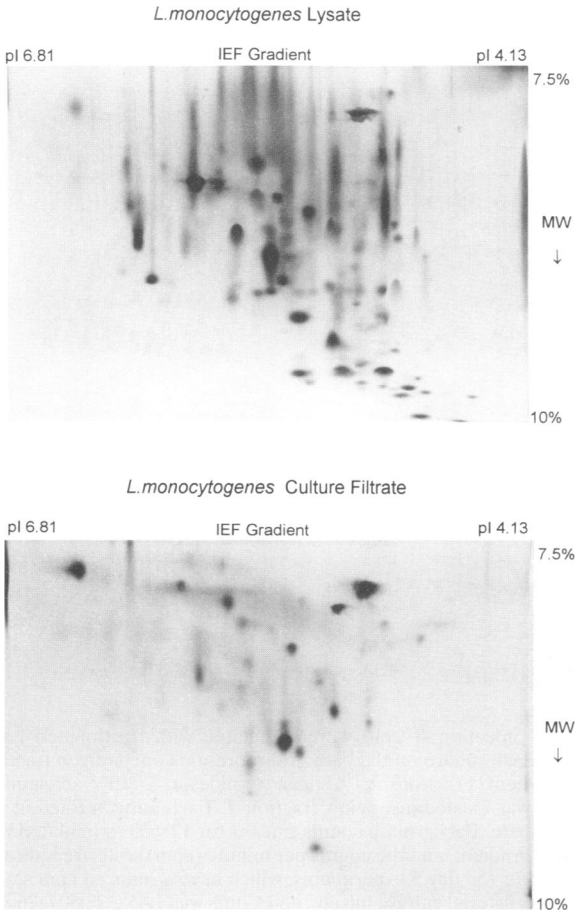

FIG. 1. Gels of *L. monocytogenes* lysate proteins (top) and culture filtrate proteins (bottom) after two-dimensional separation. One hundred micrograms of each protein preparation was separated first by isoelectric focusing (IEF) and subsequently on native polyacrylamide gradient gels (7.5 to 10%) by molecular weight (MW). Proteins were visualized with Coomassie brilliant blue and then by silver staining.

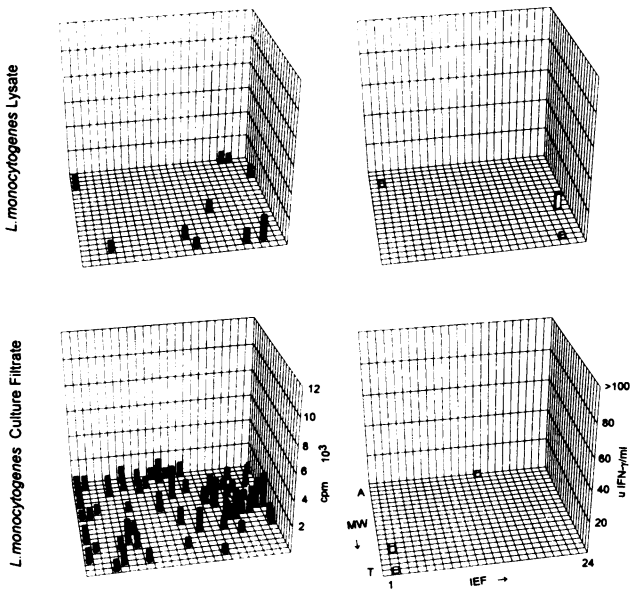

FIG. 2. Proliferation and IFN-γ profiles of T cells from naive mice. T cells were enriched from spleens and stimulated with fractionated lysate proteins (top) and fractionated culture filtrate proteins (bottom) for 4 days in vitro. Proliferation patterns are shown on the left side and IFN-γ profiles are shown on the right side for each. The size gradient and the pI gradient are indicated on the bottom right graph and are identical for all other graphs in this figure. The IFN-γ concentration scale is also given in the bottom right graph, and the radioactivity scale is indicated in the bottom left graph. Further details are given in the legend to Fig. 3. Proliferation and IFN-γ production with control antigens used at 1.25 μg/ml for medium alone, listerial lysate, listerial culture filtrate, and ConA were 1,399 cpm and 0.03 U/ml, 7,367 cpm and 0.23 U/ml, 4,477 cpm and 0.27 U/ml, and 81,904 cpm and 71.8 U/ml, respectively.

and Cellular Biology, Palo Alto, Calif. The biotinylated monoclonal rat anti-murine IL-10 antibody SXC-1 was purchased from Dianova. Murine recombinant IL-4 (rIL-4) was a kind gift from Sterling Winthrop, Malvern, Pa., and murine rIL-10 (COS cell supernatant) was a kind gift of A. Sher and I. Oswald, National Institutes of Health, Bethesda, Md. The detection limits of the ELISAs were 0.8 to 1.5 U of IFN-γ per ml, approximately 1.5 U of IL-10 per ml, and 6.3 to 12.5 U of IL-4 per ml.

RESULTS

Proliferative responses of T lymphocytes during primary infection are directed against a multitude of antigens. *L. monocytogenes* lysates (soluble somatic proteins) and culture filtrate concentrates of growing bacteria (secreted proteins) were separated by two-dimensional gel electrophoresis. Although many proteins seem to be present in both preparations, distinct secreted proteins which were not retained in the cytoplasm were identified (Fig. 1). The 480 protein fractions eluted from each gel were tested directly for their capacity to stimulate the growth of specific T lymphocytes. To exclude potential mitogenic effects of any fraction, T cells from naive mice were enriched and tested. Only marginal responses to some fractions were detected by the proliferation assay and IFN-γ ELISA (Fig. 2).

Groups of three to five mice were infected with a sublethal dose of *L. monocytogenes* and killed on days 5, 7, 10, and 14 p.i. in order to assess T-cell responses during acute- and late-phase listeriosis. T cells were enriched from the spleens and stimulated with fractions of listerial lysates or culture filtrates. Proliferation profiles are shown in Fig. 3. On day 5, T cells responded to somatic proteins only weakly but already recognized a distinct number of secreted proteins (note the reduced scale in Fig. 3). On day 7, T cells reacted with a multitude of proteins in both fractions, but the stimulation patterns of lysate proteins and secreted proteins were different. Mainly secreted proteins with a pI of between 6 and 7 and of high molecular weight were recognized by T cells, whereas stimulatory somatic proteins were more acidic and slightly smaller. On days 10 and 14 p.i., only marginal proliferative responses occurred in response to fractionated proteins of both antigen preparations. Our results indicate that a multitude of antigens rather than one or a few dominant antigens are recognized by *L. monocytogenes*-reactive T cells.

Secondary infection does not lead to selection of a limited number of antigen-specific T-cell clones. So far, memory immune responses to *L. monocytogenes* at very late time points have not been analyzed. Therefore, groups of three to four mice were infected sublethally and challenged 14 weeks later with *L. monocytogenes* or saved as control immune mice.

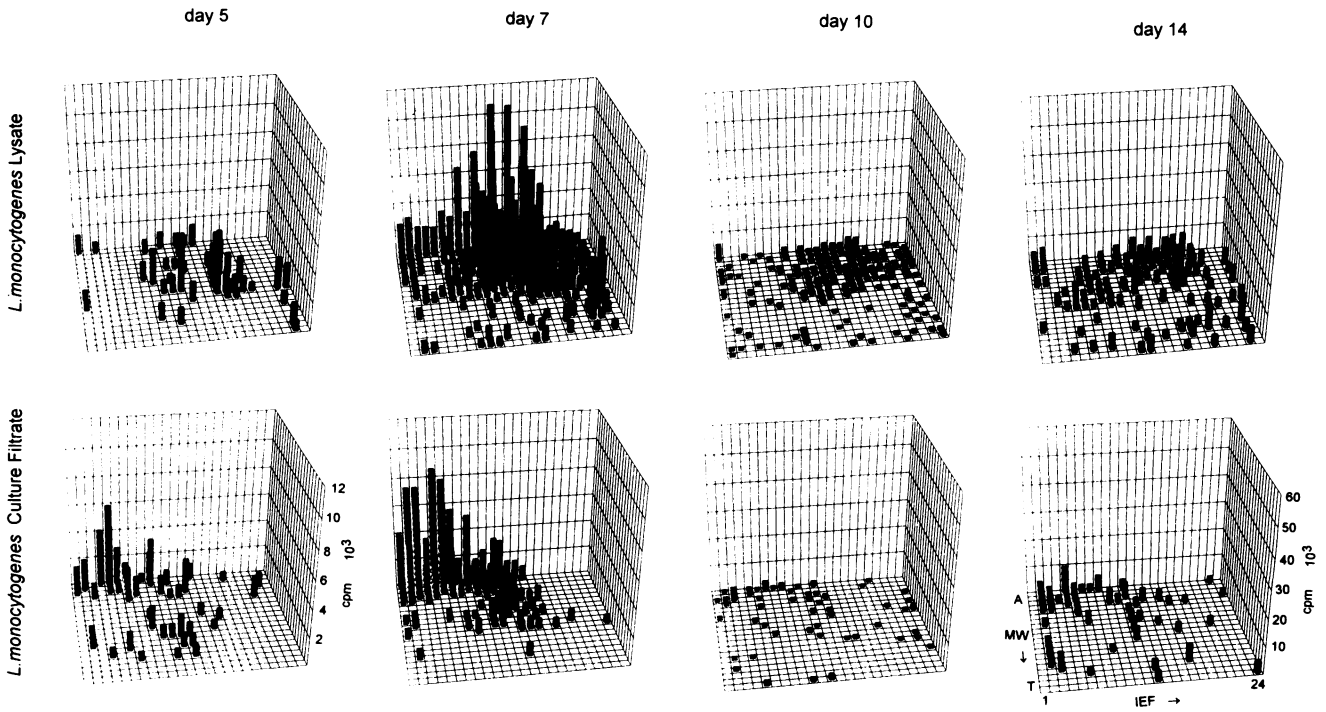

FIG. 3. Proliferation patterns of *L. monocytogenes*-reactive T lymphocytes after infection. T cells were stimulated with fractionated lysate proteins (top) and fractionated culture filtrate proteins (bottom) for 4 days in vitro. Each square on the base grid represents one antigen fraction. Fraction numbers 1 (pI 6.81) to 24 (pI 4.13) mark the isoelectric focusing (IEF) gradient. Fractions A (7.5% acrylamide) to T (10% acrylamide) indicate the molecular weight (MW) gradient. The bromophenol blue marker front was located just below fraction T. Each antigen fraction was tested in duplicates. Uptake of [^{3}H]thymidine by growing T cells is shown on the ordinate. Background counts (means for 12 wells stimulated with medium alone) were subtracted from proliferation values. The MW gradient, the IEF gradient, and the counts per minute (cpm) scale are indicated in the bottom right graph and are identical for all other graphs in this figure (except for the day 5 experiments, which have a reduced cpm scale). Proliferation with control antigens used at 1.25 μg/ml for medium alone, listerial lysate, listerial culture filtrate, and ConA was 1,458, 22,507, 26,822, and 156,629 cpm, respectively, on day 5; 12,259, 73,371, 69,044, and 64,832 cpm, respectively, on day 7; 2,021, 15,306, 13,913, and 172,109 cpm, respectively, on day 10; and 7,132, 31,722, 30,641, and 123,459 cpm, respectively, on day 14.

Because secondary listeriosis is characterized by rapid onset of immune responses, animals were killed on days 2, 4, and 7 after challenge infection and T cells were prepared (Fig. 4). Only marginal T-cell responses to fractionated listerial proteins were observed before or 2 days after reinfection. At day 4 after reinfection, various antigen fractions from both somatic and secreted listerial proteins were recognized, but these patterns appeared diffuse. Maximum T-cell responses were seen on day 7, when the recognition patterns—at least of the secreted antigens—were similar to those seen during primary infection. However, the proliferative capacity of T cells from secondarily infected animals was reduced.

To determine whether this finding was characteristic for memory immune responses or influenced by age as well, the following control animals were tested for T-cell recognition of fractionated somatic and secreted proteins: (i) naive 6-month-old mice, (ii) 6-month-old mice 7 days after primary infection with *L. monocytogenes*, and (iii) 10-week-old naive mice. Spleen cells from naive mice and T cells from infected six-month-old animals showed marked growth reduction in response to fractionated listerial proteins compared with cells from 10-week-old animals (data not shown), indicating that their reduced proliferative capacity was influenced, at least in part, by age.

To verify whether *L. monocytogenes* persisted in the spleen or liver, organ homogenates were inoculated into tryptic soy broth at various times between primary and secondary infection, but at no time after day 14 was bacterial growth detected. Furthermore, *L. monocytogenes* could not be isolated from the spleens of immune animals 2 days after reinfection, indicating that bacteria had already been cleared (data not shown). We conclude from these experiments that reinfection of immune animals with *L. monocytogenes* caused rapid reactivation of specific memory cells. T cells obtained from such mice responded to virtually the same protein fractions which were recognized during primary infection.

IFN-γ secretion patterns correlate with T-cell proliferation profiles. During primary infection, IFN-γ secretion paralleled T-cell growth in vitro (Fig. 5). Secreted proteins had induced IFN-γ production already by day 5, whereas somatic proteins were not yet stimulatory. At day 7, enormous amounts of IFN-γ were secreted in response to both somatic and secreted antigen fractions. By day 10, production of IFN-γ in response to fractionated listerial proteins had almost ceased, although some responses were still detectable with listerial lysates. During secondary infection, IFN-γ secretion also paralleled T-cell growth (Fig. 6). With fractionated somatic or secreted antigens, a specific IFN-γ response pattern was not observed before day 4. At days 4 and 7 after reinfection, higher concentrations of IFN-γ were produced in response to secreted antigens than to somatic antigens. In comparison with the profiles of primary T-cell responses, IFN-γ concentrations were reduced during secondary infection. Presumably, this decrease reflected the impact of age, because lower amounts of

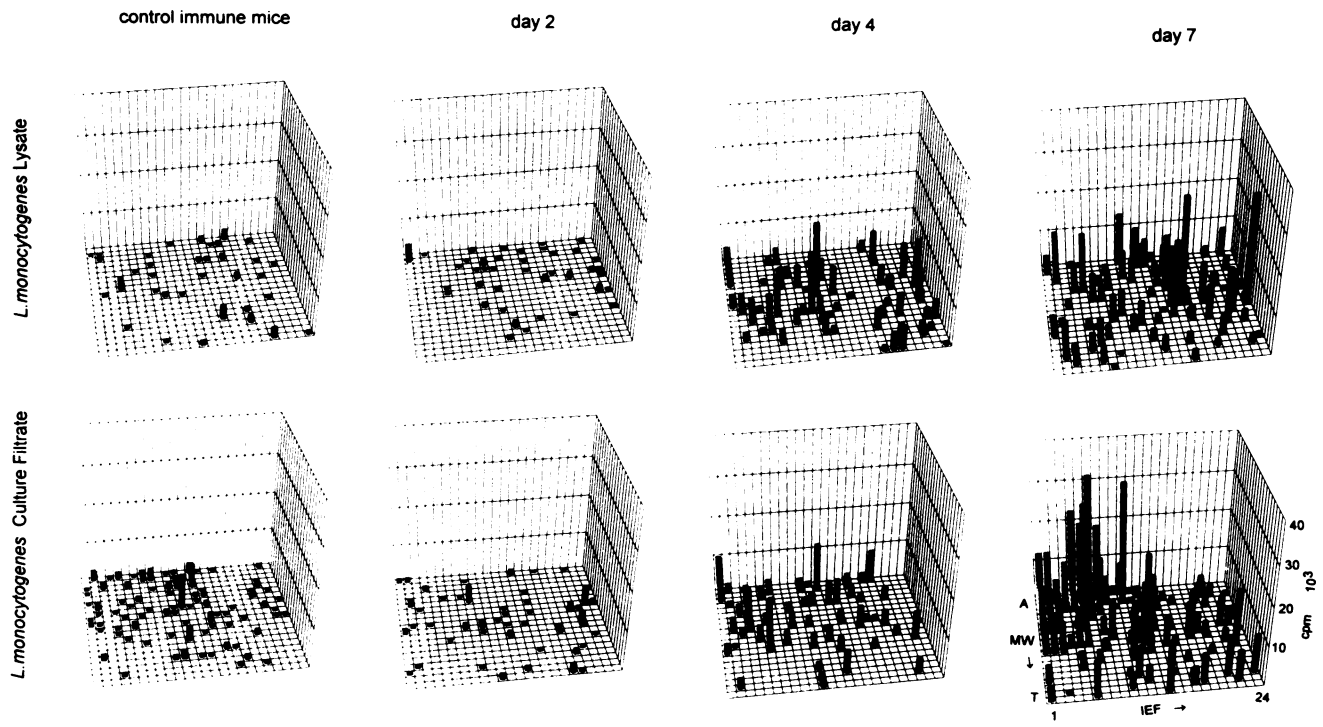

FIG. 4. T-cell proliferation patterns of immune animals after secondary infection. Mice were challenged 14 weeks after the first infection with 10^5 *L. monocytogenes* except for control immune animals. T cells were stimulated with fractionated listerial lysate proteins (top) or fractionated culture filtrate proteins (bottom) for 6 days in vitro. The MW gradient, the IEF gradient, and the cpm scale are indicated in the bottom right graph and are identical for all other graphs in this figure. Further details are given in the legend to Fig. 3. Proliferation with control antigens used at 1.25 μg/ml for medium alone, listerial lysate, listerial culture filtrate, and ConA was 367, 6,949, 5,856, and 143,572 cpm, respectively, for control immune mice. For reinfected mice, proliferation was 539, 2,360, 2,933, and 117,902 cpm, respectively, on day 2; 7,502, 14,271, 10,683, and 229,905 cpm, respectively, on day 4; and 6,799, 21,718, 26,335, and 119,282 cpm, respectively, on day 7.

IFN-γ were also observed in the age-matched controls described before. T cells from control immune mice produced large amounts of IFN-γ after in vitro stimulation with unfractionated listerial antigens, while T cells from naive mice gave only negligible responses.

IL-4 and IL-10 are not detectable in response to fractionated listerial proteins. In contrast to IFN-γ, the concentrations of IL-4 and IL-10 in culture supernatants after stimulation of T cells with either unfractionated control antigens or fractionated listerial proteins were below the detection limit of the ELISAs (data not shown).

DISCUSSION

This study is part of a comprehensive approach to characterizing the repertoire of murine T-cell responses to somatic and secreted protein antigens of *L. monocytogenes*. Separation by two-dimensional electrophoresis resulted in 480 protein fractions. It is, however, unlikely that each fraction contained only one distinct protein. Rather, some fractions probably contained several proteins with similar migration properties, and certain proteins may be contained in several adjacent fractions. None of the fractions was mitogenic, and we have no reason to assume that any of the fractions was cytotoxic, but this cannot be formally excluded. Although T lymphocytes represented only 30 to 45% of the purified cell population used for proliferation assays, remaining B cells were regarded as irrelevant because it has been demonstrated before that both proliferation and production of IFN-γ with *L. monocytogenes* antigens are completely abrogated by depletion of T cells in vitro (17, 18). Consistent with this notion, considerable amounts of IL-4 are required for B-cell proliferation in vitro (29), and as mentioned before, the concentration of IL-4 in our system was below the detection limit.

It is known that after intravenous infection with *L. monocytogenes*, more than 90% of the organisms are taken up by liver and spleen cells within the first 10 min p.i. and a high proportion of the bacteria are eliminated during subsequent hours (23). Over the next 3 to 4 days, the remaining organisms multiply continuously in host cells until specific T lymphocytes ultimately eradicate them. It can be assumed that three waves of listerial proteins provide antigens for the protective immune response. Very early after infection, a large amount of somatic proteins should become accessible to the immune system, followed by secreted proteins from growing organisms, until finally the bacteria are lysed and somatic proteins dominate again. Yet we found an exclusive focus of the early T-cell response on secreted protein antigens. This is probably because (i) inflammatory phagocytes are inefficient antigen-presenting cells and (ii) T-cell activation requires 3 to 4 days (15). T cells responded to secreted protein fractions from day 5 of primary listeriosis on, as measured by proliferation patterns and IFN-γ secretion. By day 7 p.i., somatic and secreted proteins were recognized, and high concentrations of IFN-γ were produced in response to both antigen types. Although stimulation with somatic and secreted antigens caused different proliferation patterns, in both cases a multitude of proteins were recognized rather than only one or a few

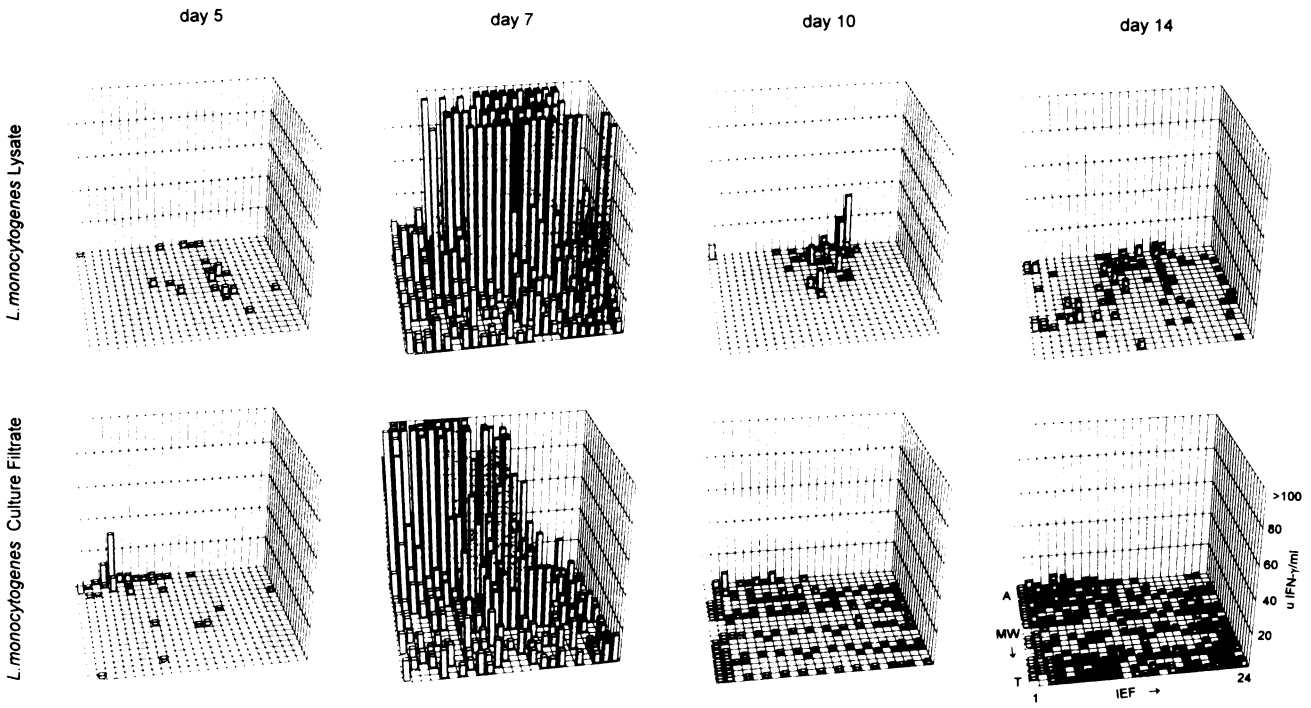

FIG. 5. IFN-γ profiles of *L. monocytogenes*-reactive T lymphocytes during primary infection. Supernatants from the T-cell cultures used to obtain the data in Fig. 3 were analyzed by double sandwich ELISA for IFN-γ. The MW gradient, the IEF gradient, and the IFN-γ concentration scale are shown in the bottom right graph and are identical for all other graphs in this figure. Further details are given in the legend to Fig. 3. IFN-γ concentrations with control antigens medium alone, listerial lysate, listerial culture filtrate, and ConA were at 0.5, >100, 73.9, and >100 U/ml, respectively, on day 5; 4.6, 43.3, 49.8, and 42.8 U/ml, respectively, on day 7; 0.2, 59.6, 45.9, and 75.9 U/ml, respectively, on day 10; and 0.3, 45.6, 44.1, and 36.6 U/ml, respectively, on day 14.

dominant antigens. Numerous secreted proteins as well as the smaller somatic proteins were not or only weakly stimulatory. Although identical amounts of proteins were loaded on isoelectric focusing gels and protein fractions were diluted with the same volumes of medium, we are aware that there is no formal standardization of the protein concentration of individual fractions and that protein quantity can influence proliferation and cytokine profiles. However, when compared with the two-dimensional PAGE gels, there was almost no correlation between the size of protein spots and fractions recognized by T cells. This finding argues against a causal relationship between abundance and antigenic dominance of listerial proteins.

Of the hundreds of *L. monocytogenes* proteins, only a few have been studied in detail (2), and so far only two types of polypeptides have been defined as T-cell antigens: (i) listeriolysin O, a hemolysin which allows escape of bacteria into the cytoplasm, representing an important virulence factor (3, 4), and (ii) peptides containing the *N*-formyl-methionine sequence, which encodes protein secretion in procaryotes (21, 31). Both antigens stimulated the production of $CD8^+$ T cells. Internalin and p60, adhesion molecules of *L. monocytogenes* that promote entry into host cells (8, 20), have been characterized as virulence factors, but their role as T-cell antigens has not yet been established. When purified listeriolysin and p60 were tested in proliferation assays, T cells from *L. monocytogenes*-infected mice strongly responded to both proteins (data not shown), but as demonstrated here, a multitude of other listerial proteins was also recognized during listeriosis. Our data are consistent with two earlier reports that T cells from *L. monocytogenes* EGD (listeriolysin positive)-immunized mice are equally well stimulated in vitro by various *Listeria* strains independent of listeriolysin expression or virulence (5, 14).

Unexpectedly, T-cell responses to fractionated listerial proteins in terms of proliferation and IFN-γ secretion were almost completely abrogated by days 10 and 14 p.i. As reported previously, protective T lymphocytes constitute a short-lived population of dividing cells which are present in the spleen until day 14 p.i. (28). Moreover, evidence has been presented that the presence of live listeriolysin-positive *L. monocytogenes* is necessary for IFN-γ production in the infected host (33). It is likely that lower levels of IFN-γ and of surface MHC class II molecules on macrophages (as a consequence of bacterial elimination) cause a rapid decline in the frequency of antigen-specific T cells. Alternatively, immunoregulatory cytokines could promote downregulation of the immune response after bacterial elimination. However, T-cell-derived IL-4 and IL-10, which are both supposed to antagonize the differentiation of TH1 cells, were not found in vitro at this time point.

Secondary infection with *L. monocytogenes* caused reactivation of antigen-specific memory T cells and probably also priming of naive T cells (9). T-cell stimulation by listerial protein fractions was not detected before day 4 after challenge infection, and maximum proliferation occurred on day 7, when antigen recognition profiles similar to those found during primary infection were seen. Similarly, IFN-γ production correlated with T-cell proliferation. T cells from control immune mice (which had not been reinfected) responded to unfractionated listerial antigens by secreting large amounts of IFN-γ in vitro but failed to react with fractionated listerial proteins. We conclude that memory T cells were present in low

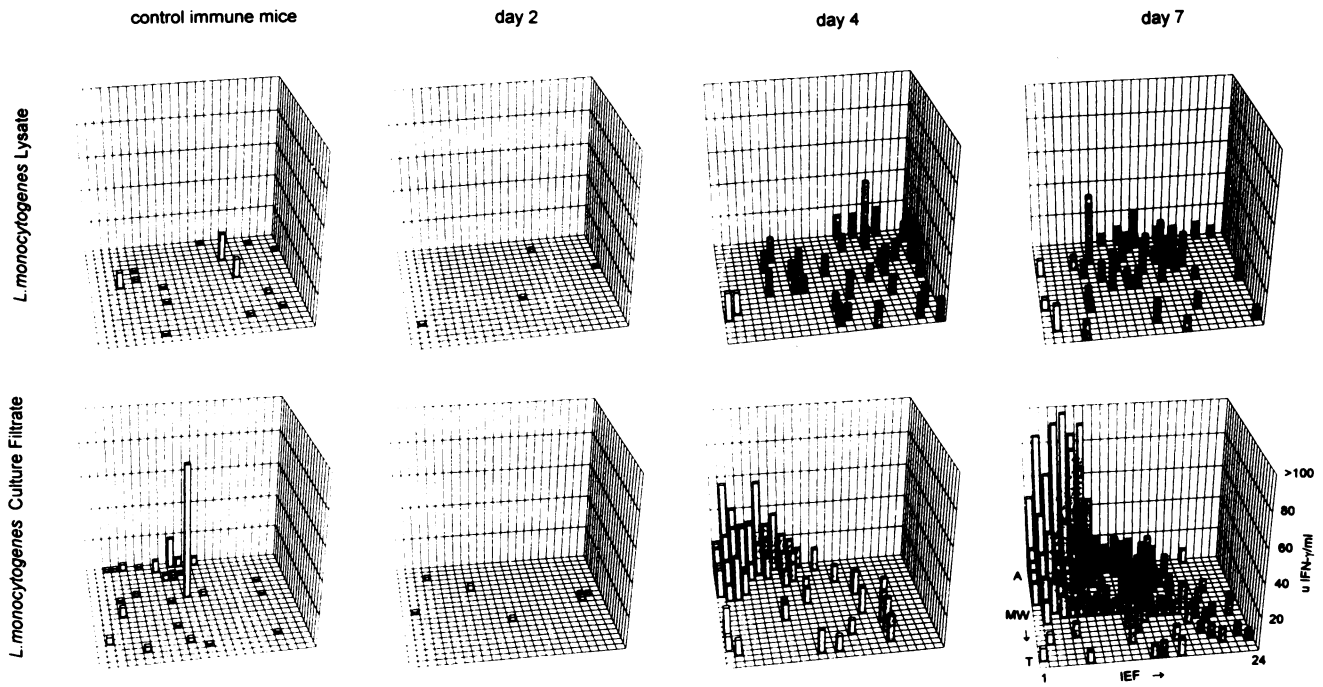

FIG. 6. IFN-γ profiles of T cells from immune mice after challenge infection with *L. monocytogenes*. Supernatants from the T-cell cultures used to obtain the data shown in Fig. 4 were analyzed by double sandwich ELISA for IFN-γ. The MW gradient, the IEF gradient, and the IFN-γ concentration scale are shown in the bottom right graph and are identical for all other graphs in this figure. Further details are given in the legend to Fig. 3. IFN-γ concentrations with control antigens medium alone, listerial lysate, listerial culture filtrate, and ConA were 0.2, 64.4, 22.3, and 52.4 U/ml, respectively, for control immune mice. For reinfected mice, IFN-γ concentrations were 0.3, 2.8, 6.8, and 50.9 U/ml, respectively, on day 2; 16.6, >100, >100, and 77.8 U/ml, respectively, on day 4; and 5.7, >100, 98.8, and >100 U/ml, respectively, on day 7.

numbers in the spleens of immune animals but that it took 4 days after challenge for them to reach levels high enough to produce a detectable response to fractionated antigens. As observed during primary infection, memory T cells were not restricted to a few dominant antigens; rather, they were specific for a large panel of stimulatory antigens. Since *L. monocytogenes* organisms were rapidly eliminated after secondary infection, one could expect that T-cell responses against somatic antigens prevailed. However, secreted proteins were preferentially recognized. Although rapid bacterial clearance from the spleens and livers of immune mice has been reported before (28), some *L. monocytogenes* organisms could have survived in a so far unidentified niche.

Although immunity to secondary listeriosis is generally thought to be long lasting, most experiments reported thus far have been performed 3 to 4 weeks after primary infection, soon after nonspecific macrophage activation has subsided. Secondary listeriosis is characterized by the rapid onset of antibacterial mechanisms (7, 26), and this response is probably driven by memory T cells. In the present study, mice were challenged with *L. monocytogenes* as late as 14 weeks after primary infection in order to analyze the antigen recognition patterns of memory T cells. In vitro proliferation and IFN-γ production by T cells were markedly influenced by the age of the mice. Nevertheless, none of the challenged mice succumbed to reinfection with high inocula, and bacteria were eliminated rapidly from spleens, although a formal survival experiment was not performed.

Our studies have been aimed at the identification of dominant T-cell antigens of intracellular bacteria. Yet in this and three previous studies (6, 10, 35), we repeatedly found that a great variety of proteins from *L. monocytogenes*, *Mycobacterium tuberculosis*, and *Mycobacterium leprae* are recognized by antigen-specific T cells rather than only a few dominant antigens. There is also increasing evidence for a major contribution of secreted mycobacterial proteins to protection of mice against challenge with virulent *M. tuberculosis* (reviewed in reference 30). Therefore, subunit vaccines or recombinant bacteria expressing one or a few dominant antigens may be inefficient inducers of protective immunity against intracellular bacteria, and live attenuated bacteria which have most protein antigens in common with the targeted pathogen may be required. Gene deletion mutants which have the vast majority of T-cell antigens of the homologous pathogen but lack the crucial virulence factors could provide successful novel means for actively directing the immune system to the development of protective memory (13).

ACKNOWLEDGMENTS

This work received financial support from SFB 322, DFG grant Ka 573/3-1, and Landesschwerpunkt "Chronic Infectious Diseases of Medical Relevance" to S.H.E.K. The project is part of the doctoral thesis of S.D. and was supported by a grant from the Graduate College "Biomolecular Medicine" at the University of Ulm.

We are grateful to G. Adolf, R. L. Coffman, W. Goebel, J. Langhorne, A. Sher, I. Oswald and the Ernst-Boehringer-Institut as well as to Sterling Winthrop for generously providing reagents. The excellent technical support of A. Zobywalski and K. Tell and the valuable help of F. Russ during the initial phase of the project are gratefully acknowledged. Many thanks to I. Flesch, L. Mertin, and A. Wand-Württenberger for reading the manuscript and for helpful suggestions.

REFERENCES

1. **Bancroft, G. J.** 1993. The role of natural killer cells in innate resistance to infection. Curr. Opin. Immunol. **5**:503–510.
2. **Beattie, I. A., B. Swaminathan, and H. K. Ziegler.** 1991. Cloning and characterization of T-cell-reactive protein antigens from *Listeria monocytogenes*. Infect. Immun. **58**:2792–2803.
3. **Berche, P., J.-L. Gaillard, C. Geoffroy, and J. E. Alouf.** 1987. T cell recognition of listeriolysin O is induced during infection with *Listeria monocytogenes*. J. Immunol. **139**:3813–3821.
4. **Bouwer, H. G. A., C. S. Nelson, B. L. Gibbins, D. A. Portnoy, and D. J. Hinrichs.** 1992. Listeriolysin O is a target of the immune response to *Listeria monocytogenes*. J. Exp. Med. **175**:1467–1471.
5. **Brocke, S., T. Chakraborty, I. Mohasseb, H. Reichert, O. Lombardi, H. Hahn, and M. Mielke.** 1992. Protective immunity and granulomatous inflammation is mediated *in vivo* by T cells reactive to epitopes common to avirulent and listeriolysin-negative mutants of *Listeria monocytogenes*. Cell. Immunol. **140**:42–53.
6. **Daugelat, S., H. Gulle, B. Schoel, and S. H. E. Kaufmann.** 1992. Secreted antigens of *Mycobacterium tuberculosis*: characterization with T lymphocytes from patients and contacts after two-dimensional separation. J. Infect. Dis. **166**:186–190.
7. **Ehlers, S., M. E. A. Mielke, T. Blankenstein, and H. Hahn.** 1992. Kinetic analysis of cytokine gene expression in the livers of naive and immune mice infected with *Listeria monocytogenes*. J. Immunol. **149**:3016–3022.
8. **Gaillard, J.-L., P. Berche, C. Frehel, E. Gouin, and P. Cossart.** 1991. Entry of *L. monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. Cell **65**:1127–1141.
9. **Gray, D.** 1993. Immunological memory: a function of antigen persistence. Trends Microbiol. **1**:39–42.
10. **Gulle, H., B. Schoel, S. Chiplunkar, S. Gangal, M. S. Deo, and S. H. E. Kaufmann.** 1992. T cell responses of leprosy patients and healthy contacts towards separated protein antigens of *Mycobacterium leprae*. Int. J. Lepr. **60**:44–53.
11. **Gulle, H., B. Schoel, and S. H. E. Kaufmann.** 1990. Direct blotting with viable cells of protein mixtures separated by two-dimensional gel electrophoresis. J. Immunol. Methods **133**:253–261.
12. **Hahn, H., and S. H. E. Kaufmann.** 1981. The role of cell-mediated immunity in bacterial infections. Rev. Infect. Dis. **3**:1221–1250.
13. **Hess, J., and S. H. E. Kaufmann.** 1993. Vaccination strategies against intracellular microbes. FEMS Immunol. Med. Microbiol. **7**:95–104.
14. **Kaufmann, S. H. E.** 1984. Acquired resistance to facultative intracellular bacteria: relationship between persistence, cross-reactivity at the T-cell level, and capacity to stimulate cellular immunity of different *Listeria* strains. Infect. Immun. **45**:234–241.
15. **Kaufmann, S. H. E.** 1986. Enumeration of *Listeria monocytogenes*-reactive L3T4$^+$ T cells activated during infection. Microb. Pathog. **1**:249–260.
16. **Kaufmann, S. H. E.** 1993. Immunity to intracellular bacteria. Annu. Rev. Immunol. **11**:129–163.
17. **Kaufmann, S. H. E., H. Hahn, R. Berger, and H. Kirchner.** 1983. Interferon-γ production by *Listeria monocytogenes*-specific T cells active in cellular antibacterial immunity. Eur. J. Immunol. **13**:265–268.
18. **Kaufmann, S. H. E., M. M. Simon, and H. Hahn.** 1982. Regulatory interactions between macrophages and T-cell subsets in *Listeria monocytogenes*-specific T-cell activation. Infect. Immun. **38**:907–913.
19. **Kongshavn, P. A. L.** 1986. Genetic control of resistance to *Listeria* infection. Curr. Top. Microbiol. Immunol. **124**:67–86.
20. **Kuhn, M., and W. Goebel.** 1989. Identification of an extracellular protein of *Listeria monocytogenes* possibly involved in intracellular uptake by mammalian cells. Infect. Immun. **57**:55–61.
21. **Kurlander, R. J., S. M. Shawar, M. L. Brown, and R. R. Rich.** 1992. Specialized role for a murine class I-b MHC molecule in procaryotic host defense. Science **257**:678–679.
22. **Locksley, R. M.** 1993. Interleukin 12 in host defense against microbial pathogens. Proc. Natl. Acad. Sci. USA **90**:5879–5890.
23. **Mackaness, G. B.** 1962. Cellular resistance to infection. J. Exp. Med. **116**:381–406.
24. **Mielke, M. E. A., G. Niedobitek, H. Stein, and H. Hahn.** 1989. Acquired resistance to *Listeria monocytogenes* is mediated by Lyt-2$^+$ T cells independently of the influx of monocytes into granulomatous lesions. J. Exp. Med. **170**:589–594.
25. **Mombaerts, P., J. Arnoldi, F. Russ, S. Tonegawa, and S. H. E. Kaufmann.** 1993. Different roles of αβ and γδ T cells in immunity against an intracellular pathogen. Nature (London) **365**:53–56.
26. **Nakane, A., T. Minagawa, M. Kohanawa, Y. Chen, H. Sato, M. Moriyama, and N. Tsuruoka.** 1989. Interactions between endogenous gamma interferon and tumor necrosis factor in host resistance against primary and secondary *Listeria monocytogenes* infections. Infect. Immun. **57**:3331–3337.
27. **North, R. J., P. A. Berche, and M. F. Newborg.** 1981. Immunologic consequences of antibiotic-induced abridgement of bacterial infection: effect on generation and loss of protective T cells and level of immunologic memory. J. Immunol. **127**:342–346.
28. **North, R. J., and J. F. Deissler.** 1975. Nature of "memory" in T-cell-mediated antibacterial immunity: cellular parameters that distinguish between the active immune response and a state of "memory." Infect. Immun. **12**:761–767.
29. **O'Garra, A., S. Umland, T. de France, and J. Christiansen.** 1988. "B-cell factors" are pleiotropic. Immunol. Today **9**:45–54.
30. **Orme, I. M., P. Andersen, and W. H. Boom.** 1993. T cell response to *Mycobacterium tuberculosis*. J. Infect. Dis. **167**:1481–1497.
31. **Pamer, E. G., C.-R. Wang, L. Flaherty, K. Fischer Lindahl, and M. J. Bevan.** 1992. H-2M3 presents a *Listeria monocytogenes* peptide to cytotoxic T lymphocytes. Cell **70**:215–223.
32. **Portnoy, D. A.** 1992. Innate immunity to a facultative intracellular bacterial pathogen. Curr. Opin. Immunol. **4**:20–24.
33. **Poston, R. M., and R. J. Kurlander.** 1992. Cytokine expression *in vivo* during murine listeriosis. J. Immunol. **149**:3040–3044.
34. **Prat, M., G. Gribaudo, P. M. Comoglio, G. Cavallo, and S. Landolfo.** 1984. Monoclonal antibodies against murine γ-interferon. Proc. Natl. Acad. Sci. USA **81**:4515–4519.
35. **Schoel, B., H. Gulle, and S. H. E. Kaufmann.** 1992. Heterogeneity of the repertoire of T cells of tuberculosis patients and healthy contacts to *Mycobacterium tuberculosis* antigens separated by high-resolution techniques. Infect. Immun. **60**:1717–1720.
36. **Schoel, B., M. Welzel, and S. H. E. Kaufmann.** Hydrophobic interaction chromatography for the purification of cytolytic bacterial toxins. J. Chromatogr., in press.
37. **Slade, S. J., and J. Langhorne.** 1989. Production of interferon-gamma during infection of mice with *Plasmodium chabaudi chabaudi*. Immunobiology **179**:353–365.
38. **Spitalny, G. L., and E. A. Havell.** 1984. Monoclonal antibody to murine gamma interferon inhibits lymphokine-induced antiviral and macrophage tumoricidal activities. J. Exp. Med. **159**:1560–1565.