

## Murine T-Cell Response to Native and Recombinant Protein Antigen of *Rickettsia tsutsugamushi*

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**A polyclonal T-cell line with TH1 characteristics was used to assess the murine cellular immune response to native and recombinant *Rickettsia tsutsugamushi* antigens. Proliferation of this T-cell line was observed in response to numerous native antigen fractions, which indicates that the murine T-helper-cell response is directed at multiple scrub typhus antigens with no apparent antigenic immunodominance. Subsequent analysis of recombinant *R. tsutsugamushi* antigens made it possible to identify a 47-kDa scrub typhus antigen (Sta47) that was stimulatory for the polyclonal T-cell line. Recombinant clones encoding 56-, 58-, and 110-kDa antigens (Sta56, Sta58, and Sta110, respectively) were unable to induce proliferation of this T-cell line. DNA sequence analysis of the cloned rickettsial insert encoding the Sta47 protein revealed the presence of four open reading frames potentially encoding proteins of 47, 30, 18, and 13 kDa. Analysis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis-separated and eluted fractions of lysates from the recombinant HB101(pRTS47B4.3) demonstrated that the fractions containing the 47-kDa protein as well as those containing proteins less than 18 kDa were stimulatory. Selected synthetic amphipathic peptides derived from the Sta47 antigen sequence identified a 20-amino-acid peptide that gave a 10-fold increase in T-cell proliferation over a control malarial peptide of similar length. Recognition of the 47-kDa antigen by a T-cell line with TH1 characteristics implicates this protein as one of potential importance in protection studies and future vaccine development.**

*Rickettsia tsutsugamushi* is an obligate intracellular bacterium and the etiologic agent of scrub typhus fever, or tsutsugamushi disease, in humans. This antigenically diverse, gram-negative organism is transmitted to humans through the bite of rickettsia-infected chiggers. Cell-mediated immunity is a major determinant in acquired resistance to *R. tsutsugamushi* (23, 36), although information regarding the diversity and specificities of antigens that are recognized by *R. tsutsugamushi*-immune T cells is extremely limited.

Murine (CD4<sup>+</sup>) T-helper lymphocytes can be divided into at least two subsets on the basis of the cytokines secreted following antigenic stimulation (26). Briefly, TH1 cells produce interleukin 2 (IL-2) and gamma interferon (IFN- $\gamma$ ), while TH2 cells produce IL-4, IL-5, and IL-10 (11, 26). The functional properties of these T-cell subsets appear to be largely a reflection of the specific cytokines produced. TH1 cells induce delayed-type hypersensitivity and activate macrophages, making these cells particularly suited to deal with intracellular organisms (5, 40, 43). TH2 cells are very efficient at providing help for antigen-specific immunoglobulin secretion, thereby enabling them to most effectively combat free-living bacteria (3, 5, 6).

IFN- $\gamma$ , the product of murine TH1 cells, has been shown to inhibit rickettsial growth in human macrophages, macrophage-like cell lines, fibroblasts, and endothelial cells in vitro (21, 27, 47, 50) and is believed to play an important role in murine resistance in vivo (12). In the murine model of scrub typhus, L3T4<sup>+</sup> Lyt2<sup>-</sup> IFN- $\gamma$ -producing T cells have been shown to adoptively transfer protection against *R. tsutsugamushi* in vivo (23). TH1 cells have also been shown to be responsible for the delayed-type hypersensitivity re-

sponse (5), which in mice with scrub typhus has been found to correlate to resistance to lethal challenge (20). The identification of T-cell stimulatory antigenic epitopes, particularly those that stimulate TH1-type cells, is an important initial step toward the development of subunit or synthetic vaccines against *R. tsutsugamushi*.

Using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE)-separated and eluted proteins of *R. tsutsugamushi*, we previously established the existence of antigens in the 18- to 35-kDa size range that were stimulatory for a T-cell line with TH1 characteristics (18). It was also demonstrated that a recombinant 22-kDa scrub typhus protein was recognized by this T-cell line and by antirickettsial antibodies. In the present report, we show that this same *R. tsutsugamushi*-specific T-cell line responds to a range of nonrecombinant scrub typhus antigens in addition to the 18- to 35-kDa region described previously. Additional analysis of this T-cell line using *Escherichia coli* lysates containing the 110-, 58-, 56-, and 47-kDa cloned recombinant *R. tsutsugamushi* antigens demonstrated that the 47-kDa *R. tsutsugamushi* antigen is capable of inducing a strong proliferative response in the TH1-like scrub typhus-responsive T-cell line. Examination of selected, synthesized amphipathic peptides derived from the 47-kDa antigen sequence (30) resulted in the delineation of a 20-amino-acid peptide capable of stimulating the T-cell line.

### MATERIALS AND METHODS

**Mice.** Female C3H/HeJ and BALB/c mice were obtained from Jackson Laboratory (Bar Harbor, Maine) and were 6 to 16 weeks of age.

**Production and continuous culture of T-cell line.** C3H/HeJ mice were given a chronic immunizing infection by inoculating them subcutaneously with 1,000 50% minimal lethal doses (when given via the intraperitoneal route) of the Karp

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strain of *R. tsutsugamushi* contained in 0.2 ml of cold brain-heart infusion broth (20). Four weeks postimmunization, splenocytes from five immunized animals were stimulated with Karp antigen (25 µg/ml) in RPMI-complete (RPMI 1640 [M. A. Bioproducts, Walkersville, Md.] supplemented with 1% fresh glutamine, 50 µg of gentamicin per ml, 10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid] buffer, and  $5 \times 10^{-5}$  M 2-mercaptoethanol) containing 5% heat-inactivated, hybridoma-screened fetal bovine serum (FBS) (M. A. Bioproducts). The T-cell line was maintained in vitro by alternating 10-day periods of rest with 4-day periods of antigenic stimulation as described previously (18).

**Lymphocyte proliferation assay.** The ability of the T-cell line to proliferate in response to native or recombinant rickettsial antigens was determined as described previously (18). Briefly, microcultures (200 µl) containing  $1 \times 10^4$  T-cell blasts,  $5 \times 10^5$  syngeneic irradiated (3 kilorads) spleen cells, and 100 µl of antigen were stimulated in RPMI-complete containing 2.5% FBS for 72 h at 37°C in a 7% CO<sub>2</sub>-93% air atmosphere. [<sup>3</sup>H]thymidine (1 µCi per well; 6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was added for the final 6 h of culture. Cells were harvested onto glass fiber strips by using a multiple harvesting system, and the amount of incorporated radioactivity was determined by liquid scintillation counting. All experiments were done in triplicate, and data were expressed as mean uptake of [<sup>3</sup>H]thymidine ± standard error of the mean (SEM).

**Native and recombinant *R. tsutsugamushi* antigens.** Native rickettsial antigens were prepared as described elsewhere (18). Recombinant λgt11 clones expressing antigenic determinants of *R. tsutsugamushi* protein antigens were identified and isolated from genomic λgt11 libraries as described previously (31). Plaque-purified λgt11 recombinants were used to affinity purify antibodies specific for the recombinant antigens from hyperimmune serum (rabbit anti-Karp). The corresponding native full-length *R. tsutsugamushi* antigen encoded by each recombinant was identified by using these recombinant antigen-specific sera in Western blot (immunoblot) analysis of whole-cell *R. tsutsugamushi* lysates (29). The recombinant clones HB101(pRTS110C5.2), HB101(pRTS58H2.9), HB101(pRTS56H2.3), and HB101(pRTS47C8.4) or HB101(pRTS47B4.3) produced scrub typhus antigens Sta110, Sta58, Sta56, and Sta47, respectively, which appeared to have the same molecular weights as the native rickettsial proteins (29, 31). Recombinant lysates were prepared by suspending late-log-phase culture pellets of the plasmid-containing organisms in RPMI-complete and lysing them with a French press. The French press lysates were immediately frozen. Recombinant lysates were analyzed for protein content by using a modification of the Lowry method (33). Lysates were adjusted to equivalent protein concentrations and sterilized by irradiation (300 kilorads) prior to use. Recombinant antigens were diluted in RPMI-complete for use in lymphocyte proliferation assays.

**Western blotting.** Rabbit anti-*R. tsutsugamushi* antiserum was prepared from a rabbit inoculated with gradient-purified whole *R. tsutsugamushi* (strain Karp). This serum was exhaustively absorbed with *E. coli* for use in Western blot analysis of recombinant organisms (31). SDS-PAGE and Western blotting of rickettsial polypeptides were performed as previously described (4, 28). Staphylococcal protein A conjugated with alkaline phosphatase (Cappel, Organon Teknika Corp., West Chester, Pa.) was used to detect the antibody bound to antigens in the Western blot assay. Alkaline phosphatase-conjugated probes were developed

with fast red TR salt and naphthol AS-MX phosphate as previously described (37).

**Electroelution of *R. tsutsugamushi* antigens.** Lysates of *R. tsutsugamushi* and recombinant bacteria HB101(pRTS47C8.4) and HB101(pBR322) (control) were electrophoresed and eluted from SDS-polyacrylamide gels. The eluted fractions were examined for the ability to stimulate the *R. tsutsugamushi*-reactive T-cell line as previously described (18).

**Cell surface phenotype analysis.** Surface phenotype of the C3H/HeJ T-cell line was determined by analyzing the binding of monoclonal antibodies by fluorescence flow cytometry. Antigen-stimulated T cells were washed twice in Hanks balanced salt solution, and viable cells were enumerated by trypan blue exclusion and adjusted to 10<sup>6</sup> cells per ml in phosphate-buffered saline (PBS) containing 2% FBS and 0.1% sodium azide (PBS-azide). Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody anti-Thy-1.2, anti-Lyt-2, or anti-L3T4 (Becton Dickinson Immunocytometry Systems, Lincoln Park, N.J.) was added to 1-ml aliquots of cells. Mixtures were incubated for 1 h at 4°C and then washed three times with PBS-azide. Cells stained with directly FITC-conjugated monoclonal antibodies were fixed in 1% paraformaldehyde in PBS and stored at 4°C in the dark. Anti-L3T4-treated cells were further stained with FITC-labeled goat anti-mouse immunoglobulin (Becton Dickinson Immunocytometry Systems) as described above, washed, and fixed in 1% paraformaldehyde. Samples (10<sup>4</sup> cells) were analyzed for fluorescence on a log scale with a Facscan 440 fluorescence-activated cell sorter (Becton Dickinson Immunocytometry Systems). Control samples consisted of unstained cells and cells stained with FITC-labeled goat anti-mouse immunoglobulin.

**Measurement of cytokine production.** Cytokine bioassays to detect IL-2 and IL-3 production were performed with cytokine-dependent cell lines (19). The IL-3-dependent cell line DA1 was kindly provided by J. Ihle, Frederick Cancer Research Center, Frederick, Md. The IL-2-dependent cell line CTLL was the kind gift of Ethan Shevach, National Institutes of Health, Bethesda, Md.

IFN-γ production was determined by using a murine IFN-γ double-sandwich enzyme-linked immunosorbent assay (ELISA) (7). Briefly, 1 µg of purified murine anti-IFN-γ monoclonal antibody (Lee Biomolecular Research Inc., San Diego, Calif.) contained in 100 µl of 50 mM Tris-HCl-50 mM NaCl at pH 8 was added to each well of a 96-well plate (Immulon II; Dynatech Laboratories Inc., Torrance, Calif.) and allowed to bind overnight at 4°C. Unabsorbed monoclonal antibody was removed, and 200 µl of casein buffer containing 7.5 mM Tris, 2% casein, and 0.2% sodium azide (pH 7.5) was added to each well for 2 h at room temperature. Plates were washed five times with PBS-Tween (30) prior to the addition of samples (100 µl). Recombinant murine IFN-γ (AMGen Biologicals, Thousand Oaks, Calif.) with a specific activity of  $\geq 10^7$  U/mg was used as a standard. Primary culture supernatant obtained from conalbumin-stimulated D10 cells was used as a negative control. D10 cells are conalbumin-specific T cells of the TH2 subclass and do not produce IFN-γ (15). Plates were incubated for 3 h at 37°C and then washed prior to the addition of 100 µl of a 1:1,000 dilution of polyclonal rabbit anti-mouse IFN-γ, which was generously provided by G. Spitalny (Bristol-Myers Co., Wallingford, Conn.). Following a 2-h incubation at room temperature, the plates were washed five times with PBS-Tween, and 100 µl of affinity-purified horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Boeh-

TABLE 1. Synthetic peptides used in this study

Peptide <sup>a</sup>	Sequence <sup>b</sup>
pepSta47(11-33)	VIAVVAVHAI FNSV FQVMQNQYA
pepSta47(41-60)	AVKVI SE PGLR FKV P FV QNV
pepSta47(81-100)	DGKRVI VNAFAKFKIIDPIT
pepSta47(91-110)	AKFKIIDPITFFKTVTNHNG
pepSta47(105-125)	VTNHNGVKIRLNKTIESAMRK
pepSta47(218-238)	LAEAYKQAKILEGEGVAEASH
pepSta47(236-260)	ASHIYNSVYSRI PRFYR FYQSLLTY
pepSta47(242-269)	SVYSRI PRFYR FYQSLLTYSKVLRKDDT
K3	KKCKYNATKAE
CV2	CYGGGDRADGQPAGDRADGQPA

<sup>a</sup> Peptides with pepSta47 prefix represent amphipathic peptides derived from the sequence of Sta47. Peptides K3 and CV2 are control peptides derived from the sequence of the malarial circumsporozoite protein and were kindly provided by Lynnette Smith, Department of Bacterial Diseases, Walter Reed Army Institute of Research, Washington, D.C.

<sup>b</sup> Single-letter amino acid codes are used.

ringer Mannheim Biochemicals, Indianapolis, Ind.) diluted 1:1,000 was added for 2 h. Plates were washed five times with PBS-Tween, and 100  $\mu$ l of substrate (1 mg of *o*-phenylenediamine per ml and 0.012% hydrogen peroxide in 0.1 M sodium citrate, pH 4.5) was added. Plates were read at 490 nm on a Titertek Multiscan (Dynatech Laboratories).

**Amphipathic peptides.** The 47-kDa protein amino acid sequence (30) was analyzed by using the computer algorithm AMPHI (24) to predict segments that could best form amphipathic helices. Eight synthetic peptides (Table 1) corresponding to areas with high amphipathic indices were produced commercially. Peptides were reconstituted in sterile deionized H<sub>2</sub>O at a concentration of 1 mg/ml, frozen at -80°C, and irradiated with 300 kilorads. Peptides were diluted in RPMI-complete and examined for the ability to stimulate the T-cell line during a lymphocyte proliferation assay. Fourfold dilutions of concentrations ranging from 1.0 ng/ml to 100  $\mu$ g/ml were tested.

## RESULTS

**Proliferative response of C3H/HeJ T-cell line to native *R. tsutsugamushi* antigens.** The C3H/HeJ T-cell line was developed and maintained in vitro by stimulation with the Karp strain of *R. tsutsugamushi* as previously described (18). In order to determine the strain specificity of this line, proliferative responses to the homologous Karp antigen, heterol-

ogous Kato and Gilliam antigens, and irrelevant antigens were examined. As shown in Table 2 and previously (18), this T-cell line gave a strong proliferative response to the homologous Karp antigen as well as cross-reactive responses to the Kato and Gilliam strains of *R. tsutsugamushi*. The T-cell line did not proliferate in response to antigen diluent, an L-929 cell preparation, or *Rickettsia australis* antigen or in the absence of accessory cells (data not shown).

**Phenotypic characterization of T-cell line.** Evaluation of surface phenotypic markers on the T-cell line by fluorescence flow cytometry identified a population of cells that were 98% Thy-1.2<sup>+</sup>, 98% L3T4<sup>+</sup>, and <1% Lyt2<sup>-</sup>. This phenotype is characteristic of T cells that recognize antigen in the context of the class II major histocompatibility complex molecule. The antigenic response of the T-cell line appeared to be class II restricted, since syngeneic C3H/HeJ but not allogeneic BALB/c spleen cells could function as antigen-presenting cells (data not shown). The cytokine secretion pattern of the T-cell line was examined following antigenic stimulation. The T-cell line was found to produce IL-3, a cytokine produced by both TH1 and TH2 subsets, as well as IFN- $\gamma$  and IL-2, cytokines characteristic of the TH1 subset of murine T-helper cells (Table 2).

**Proliferative response of T-cell line to electroeluted native *R. tsutsugamushi* antigens.** Because *R. tsutsugamushi* antigens are extremely difficult to obtain in pure form, electrophoretically separated and eluted native *R. tsutsugamushi* antigens were used to determine the antigenic repertoire of the T-cell line. This technique makes it possible to identify stimulatory antigenic regions that correspond to a particular molecular size range. In this manner, it was previously demonstrated that eluted samples (undiluted) from low-molecular-mass fractions (18 to 35 kDa) of SDS-PAGE-separated whole-cell *R. tsutsugamushi* were stimulatory for this T-cell line (18). We now demonstrate that several additional gel regions stimulate reactivity following dilution of the eluted gel samples. Figure 1 shows that several fractions (depending on dilution of eluate) contain rickettsial antigens capable of stimulating the T-cell line. Previously described *R. tsutsugamushi* antigens present in the various stimulatory fractions include the 165-, 150-, and 138-kDa proteins and the Sta110 protein in fraction 1; the 72-kDa protein antigen in fraction 2; the Sta58 and Sta56 proteins in fraction 3; the Sta47 and heat-modifiable form of the Sta56 protein in fraction 4; and antigens smaller than 18 kDa in fractions 9 through 12. A vigorous T-cell response was also observed

TABLE 2. Proliferative response and cytokine production by *R. tsutsugamushi*-reactive C3H/HeJ T-cell line<sup>a</sup>

Antigen	Proliferation (cpm) <sup>b</sup>	IFN- $\gamma$ (U/ml) <sup>c,d</sup>	IL-2 (cpm) <sup>c,e</sup>	IL-3 (cpm) <sup>c,e</sup>
None	238 $\pm$ 24	7.9 $\pm$ 0.4	103 $\pm$ 21	568 $\pm$ 20
Karp	44,481 $\pm$ 5,528	1,830 $\pm$ 47.0	8,302 $\pm$ 1,543	68,256 $\pm$ 7,802
Kato	42,382 $\pm$ 5,026	2,170 $\pm$ 24.0	ND	80,620 $\pm$ 5,627
Gilliam	12,693 $\pm$ 2,660	1,530 $\pm$ 15.0	ND	73,643 $\pm$ 7,561

<sup>a</sup> All values are means  $\pm$  SEM. ND, not done.

<sup>b</sup> Data are for triplicate microcultures (200  $\mu$ l) containing T cells ( $1 \times 10^4$  per well), syngeneic irradiated antigen-presenting cells ( $5 \times 10^5$  per well), and antigen (100  $\mu$ l). Results are expressed as mean uptake of [<sup>3</sup>H]thymidine per well. Antigens of Karp, Kato, and Gilliam strains of *R. tsutsugamushi* were used at a concentration of 100  $\mu$ g of protein per ml. Negative control antigens *R. australis* (100  $\mu$ g of protein per ml) and an L-929 cell homogenate (100  $\mu$ g of protein per ml) did not elicit a proliferative response (726  $\pm$  78 and 329  $\pm$  59 cpm, respectively). The optimal antigen dose determined for each antigen preparation ranged from 25 to 100  $\mu$ g of protein per ml.

<sup>c</sup> T cells from C3H/HeJ T-cell line ( $1 \times 10^4$  per well) were cocultured (200  $\mu$ l, total volume) with syngeneic antigen-presenting cells ( $5 \times 10^5$  well) and 100  $\mu$ l of antigen diluent (None), Karp (50  $\mu$ g/ml), Kato (50  $\mu$ g/ml), or Gilliam (50  $\mu$ g/ml). Lymphokine-containing supernatants were harvested at 48 h for the IL-2 and IL-3 bioassays and at 72 h for the IFN- $\gamma$  ELISA.

<sup>d</sup> Values were obtained by linear regression on a recombinant murine IFN- $\gamma$  standard curve and represent means of triplicate samples  $\pm$  SEM.

<sup>e</sup> Uptake of [<sup>3</sup>H]thymidine in triplicate cultures of CTLL (IL-2) or DA1 (IL-3) cells supplemented with appropriate primary culture supernatant.

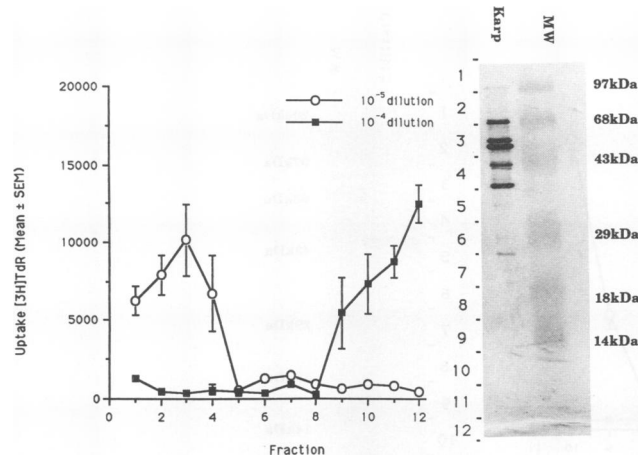


FIG. 1. Proliferative response of C3H/HeJ T-cell line to electroeluted gel fractions of *R. tsutsugamushi* Karp. Microcultures (200  $\mu$ l, total volume) of T cells ( $1 \times 10^4$  per well) were incubated with syngeneic irradiated antigen-presenting cells ( $5 \times 10^5$  per well) and various fractions (100  $\mu$ l) of L-929 Karp that had been electrophoresed on SDS-15% PAGE gels, fractionated, and electroeluted. Electroeluted samples were dialyzed extensively against PBS following electroelution and diluted to  $10^{-5}$  in RPMI-complete without FBS. Data are shown as mean [ $^3$ H]thymidine ([ $^3$ H]Tdr) uptake  $\pm$  SEM of triplicate cultures. Proliferative responses were  $17,258 \pm 3396$  cpm to a whole-cell lysate of column-purified Karp and  $424 \pm 71$  cpm to antigen diluent. A representative lane of the SDS-polyacrylamide gel prior to fractionation was electroeluted onto nitrocellulose and developed by using polyclonal rabbit anti-Karp antibodies. MW, molecular mass.

( $10^{-4}$  dilution) in fractions 9 through 12, which correspond to the region of the gel containing polypeptides smaller than 18 kDa. Uninfected L-929 cells that had been electrophoresed, fractionated, and electroeluted were not stimulatory for this T-cell line (18). These data demonstrate that the murine T-cell line recognizes a wide range of *R. tsutsugamushi* proteins. In general, T-cell recognition of eluted fractions corresponded well to the presence of antigens recognized by rabbit anti-Karp antisera; however, other antigens that stimulate T cells but are not recognized by antibody may have been present in these preparations.

**Proliferative response of T-cell line to recombinant *R. tsutsugamushi* antigens.** In order to examine the T-cell response to individual scrub typhus antigens, the *R. tsutsugamushi*-reactive T-cell line was examined for the ability to proliferate in response to extracts of recombinant organisms expressing scrub typhus antigens. *E. coli* expressing either the Sta110 [HB101(pRTS110)], Sta58 [HB101(pRTS58)], Sta56 [HB101(pRTS56)], or Sta47 [HB101(pRTS47C8.5)] protein antigens of *R. tsutsugamushi* Karp and the HB101 (pBR322) control were examined. Antigen preparations consisted of French press lysates that had been irradiated with 300 kilorads to prevent the growth of the small portion of unlysed *E. coli*. A 12-fold increase in proliferation was seen in response to a lysate of the HB101(pRTS47C8.5) recombinant compared with the HB101(pBR322) control lysate (Fig. 2). T-cell proliferation in response to this antigen followed a typical antigen dose-response curve, with maximal stimulation seen at a crude protein concentration of 1 to 10  $\mu$ g/ml. Lysates containing the Sta110, Sta58, and Sta56 recombinant proteins gave a minimal response, if any, compared with that of the HB101(pBR322) control. This lack of response may have reflected low-level antigen expression by

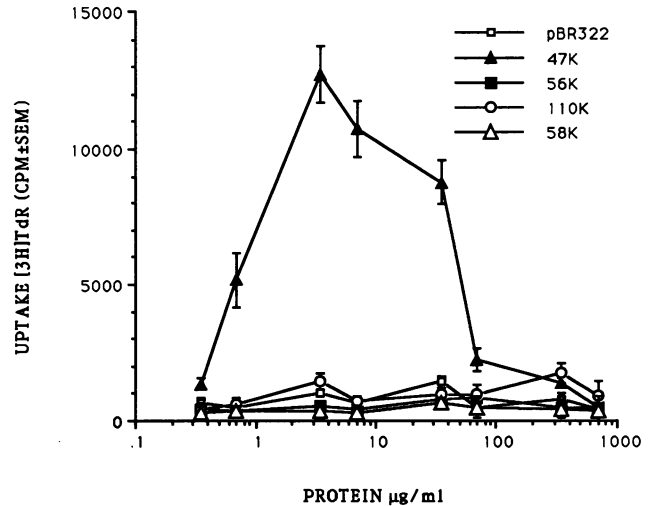


FIG. 2. Proliferative response of *R. tsutsugamushi*-reactive C3H/HeJ T-cell line to recombinant *R. tsutsugamushi* protein antigen preparations of *E. coli* HB101(pRTS47), HB101(pRTS56), HB101(pRTS110), HB101(pRTS58), or HB101(pBR322) control. Microcultures (200  $\mu$ l, total volume) of T cells ( $1 \times 10^4$  per well) were incubated with syngeneic irradiated antigen-presenting cells ( $5 \times 10^5$  per well) and various concentrations of recombinant or control lysates. Antigen preparations consisted of French press lysates that were sterilized by irradiation (300 kilorads). Data are presented as mean uptake of [ $^3$ H]thymidine ([ $^3$ H]Tdr) in counts per minute  $\pm$  SEM of triplicate cultures.

recombinants HB101(pRTS110), HB101(pRTS58), and HB101 (pRTS56) in contrast to the higher levels of expression of Sta47 by recombinant HB101(pRTS47C8.5) (30).

**Analysis of eluted fractions of recombinant *R. tsutsugamushi* antigens.** As mentioned above, T-cell proliferation was seen in response to eluted fractions of native Karp that had approximate molecular masses of 18 kDa and less (Fig. 1). The DNA sequence of the *R. tsutsugamushi* insert in pRTS47C8.5 indicated that this 8.5-kb piece of *R. tsutsugamushi* DNA contained open reading frames for proteins of 47, 30, 18, and 13 kDa (30). It was therefore possible that a protein in addition to the Sta47 protein was being expressed by this recombinant and was responsible for or contributing to the proliferative response seen previously to extracts of HB101(pRTS47C8.5). Although the Sta47 protein was the major protein expressed by this clone and recognized by polyclonal rabbit anti-Karp antiserum, additional proteins not recognized or recognized weakly by this antiserum but containing T-cell epitopes could have been present.

Lysates of clone HB101(pRTS47B4.3), which contains an internal 4.3-kb *Bgl*II fragment of pRTS47C8.5 and encodes the 47-, 30-, 18-, and 13-kDa proteins, were fractionated by SDS-PAGE followed by proliferation assays. As shown in Fig. 3, fraction 5 (1:10 dilution) of clone HB101(pRTS 47B4.3), which corresponds to the 43-kDa molecular mass marker and would contain the 47-kDa recombinant protein, elicited a proliferative response (9,101 cpm). In addition, strong proliferative responses to fractions 9, 10, and 11 of this recombinant clone were seen. Fraction 9 spans the region of the gel from 18 to 14 kDa, while fractions 10 and 11 span the region of the gel from 14 kDa to the dye front. Degradation products of the Sta47 or uncharacterized gene products from the 4.3-kb *Bgl*II fragment may account for the activities of these low-molecular-mass fractions. Dilutions of

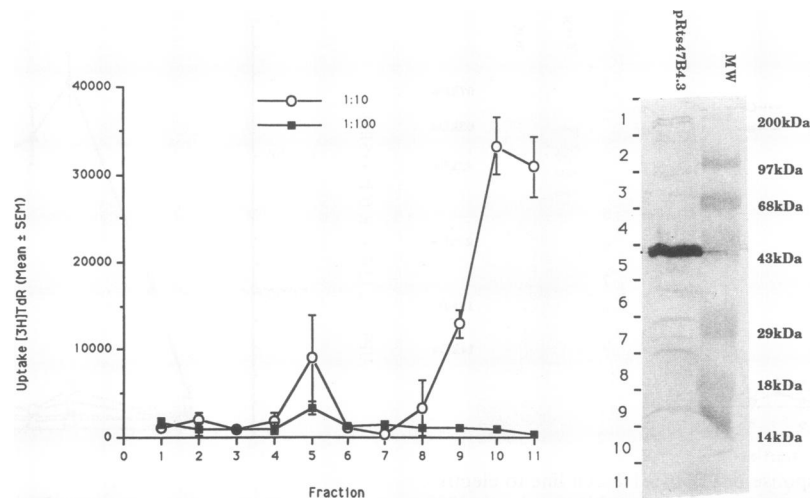


FIG. 3. Proliferative response of C3H/HeJ T-cell line to electroeluted gel fractions of HB101(pRTS47B4.3). Microcultures (200  $\mu$ l, total volume) of T cells ( $1 \times 10^4$  per well) were incubated with syngeneic irradiated antigen-presenting cells ( $5 \times 10^5$  per well) and 100  $\mu$ l of HB101(pRTS47B4.3) that had been electrophoresed on SDS-15% polyacrylamide gels, fractionated, and electroeluted. Electrophoresed samples were dialyzed extensively against PBS following electroelution and then diluted 1:10 in RPMI-complete without FBS. Data are shown as mean [ $^3$ H]thymidine ([ $^3$ H]TdR) uptake  $\pm$  SEM of triplicate cultures. The response of the T-cell line to column-purified Karp was  $17,258 \pm 3,396$  cpm, while the response to antigen diluent alone was  $424 \pm 71$  cpm. Dilutions of HB101(pBR322) that had been electrophoresed, fractionated, and electroeluted (undiluted to 1:100,000) were not stimulatory for the T-cell line (data not shown). A representative lane of the SDS-polyacrylamide gel prior to fractionation was electroeluted onto nitrocellulose and developed by using polyclonal rabbit anti-Karp antibodies. MW, molecular mass.

HB101(pBR322) that had been electrophoresed, fractionated, and electroeluted (undiluted to 1:100,000) were not stimulatory for the T-cell line (data not shown).

**Amphipathic analysis of Sta47 amino acid sequence.** The sequence of the cloned *R. tsutsugamushi* DNA of HB101 (pRTS47C8.5) was determined (30) and found to possess open reading frames for proteins of 47, 30, 18, and 13 kDa. The computer algorithm AMPHI (24), which identifies potential T-cell epitopes on the basis of the abilities of peptides to form stable amphipathic helices, was used to identify potential T-cell sites in the Sta47 protein sequence. Based on this analysis, eight peptides (Table 1) representing regions with high amphipathic scores were synthesized by commercial sources and tested for the ability to induce proliferation of the C3H/HeJ T-cell line. As shown in Table 3, peptide 81-100 (Asp-Gly-Lys-Arg-Val-Ile-Val-Asn-Ala-Phe-Ala-Lys-Phe-Lys-Ile-Ile-Asp-Pro-Ile-Thr) was capable of eliciting

a proliferative response 10 times greater than that of a control malarial peptide of similar length, suggesting that this 20-amino-acid residue peptide contains a stimulatory T-cell epitope of the 47-kDa *R. tsutsugamushi* antigen. Maximal stimulation by this peptide was seen at a peptide concentration of approximately 1  $\mu$ g/ml. The remaining Sta47 amphipathic peptides, including peptide 91-110, which has a 10-amino-acid overlap with peptide 81-100, did not induce significant proliferation of this T-cell line.

## DISCUSSION

Immunity to *R. tsutsugamushi* is dependent on the development of a cell-mediated immune response; however, the identities and natures of the antigens involved in this response are largely undefined. Like other obligate intracellular bacterial pathogens, *R. tsutsugamushi* consists of a complex mosaic of polypeptide antigens, only some of which contain T-cell epitopes capable of eliciting a protective immune response. The identification of antigens that stimulate a cell-mediated immune response and the subsequent mapping of T-cell epitopes on these antigens are important in order to achieve a better understanding of homologous and heterologous immunity to scrub typhus and are important initial steps toward the development of future vaccines.

IFN- $\gamma$ , produced by murine TH1 cells (26), is believed to be of critical importance in the protective immune response to various intracellular organisms, including *R. tsutsugamushi* (1, 2, 22). IFN- $\gamma$  inhibits rickettsial growth in human macrophages, macrophage-like cell lines, fibroblasts, and endothelial cells in vitro (21, 27, 47, 50) and is believed to play an important role in murine resistance in vivo (12). Murine IFN- $\gamma$ -producing CD4 $^+$  T cells have been shown to adoptively transfer protection against *R. tsutsugamushi* in vivo (23).

TABLE 3. Proliferative response of T-cell line to amphipathic peptides derived from Sta47<sup>a</sup>

Peptide (amino acid residues)	[ $^3$ H]thymidine uptake (cpm) at peptide concn of:			
	0.1 $\mu$ g/ml	0.4 $\mu$ g/ml	1.6 $\mu$ g/ml	6.4 $\mu$ g/ml
pepSta47(11-33)	552 $\pm$ 55	695 $\pm$ 27	654 $\pm$ 97	950 $\pm$ 3
pepSta47(41-60)	456 $\pm$ 8	538 $\pm$ 193	506 $\pm$ 272	236 $\pm$ 50
pepSta47(81-100)	196 $\pm$ 33	304 $\pm$ 53	4,621 $\pm$ 685	3,388 $\pm$ 439
pepSta47(91-110)	373 $\pm$ 31	167 $\pm$ 24	352 $\pm$ 46	632 $\pm$ 391
pepSta47(105-125)	264 $\pm$ 67	478 $\pm$ 157	285 $\pm$ 140	556 $\pm$ 72
pepSta47(218-238)	292 $\pm$ 120	467 $\pm$ 217	377 $\pm$ 90	456 $\pm$ 224
pepSta47(236-260)	367 $\pm$ 127	468 $\pm$ 197	1,258 $\pm$ 453	357 $\pm$ 74
pepSta47(242-269)	1,171 $\pm$ 72	294 $\pm$ 62	277 $\pm$ 85	440 $\pm$ 222
CV2	355 $\pm$ 110	360 $\pm$ 240	724 $\pm$ 262	284 $\pm$ 66

<sup>a</sup> T cells ( $1 \times 10^4$  per well) were incubated with syngeneic irradiated antigen-presenting cells ( $5 \times 10^5$  per well) and various concentrations of Sta47-derived peptides or CV2, a malarial circumsporozoite control peptide. Data are for triplicate cultures.

The present study used an IFN- $\gamma$ -producing TH1-like polyclonal T-cell line to characterize the murine T-cell response following an immunizing infection with *R. tsutsugamushi*. *R. tsutsugamushi* antigens eluted from SDS-polyacrylamide gels, recombinant rickettsial antigens, and synthetic peptides were examined in an effort to identify relevant antigens and individual polypeptides capable of activating *R. tsutsugamushi*-responsive T cells.

Initial experiments utilized native eluted *R. tsutsugamushi* antigens to determine the scope of the murine antigenic repertoire. These studies demonstrated that the murine cellular immune response is directed against multiple *R. tsutsugamushi* antigens with no obvious preference for a few immunodominant ones. Numerous fractions containing antigens eluted from SDS-PAGE gels were shown to stimulate the *R. tsutsugamushi*-responsive T-cell line. The stimulatory fractions in many cases contained well-characterized antigens recognized by polyclonal antisera; however, it is possible that T-cell antigens not recognized by the antiserum were present and responsible for stimulation.

The use of cloned recombinant antigens makes it possible to focus on and identify individual polypeptides important in the cellular immune response. In this manner, we previously demonstrated that the Sta22 *R. tsutsugamushi* antigen was recognized by both cellular and humoral immune mechanisms (18). The present report includes the analysis of four cloned recombinant scrub typhus rickettsial antigens (Sta110, Sta58, Sta56, and Sta47) and demonstrates that Sta47 was stimulatory for the *R. tsutsugamushi*-responsive T-cell line. The 47-kDa protein of *R. tsutsugamushi* is a major rickettsial antigen that is found in the outer membrane (45) and contains both scrub typhus group-reactive (13, 45) and strain-specific (25) B-cell epitopes.

Surprisingly, the recombinants expressing the Sta56, Sta58, and Sta110 polypeptides did not induce proliferation of the polyclonal T-cell line. Sta56 is a quantitatively major *R. tsutsugamushi* protein against which most animals and infected humans produce antibodies (14, 31). Sta56 contains both strain-specific and group-reactive epitopes (9, 13, 31, 41, 45). Sta58 is also a quantitatively major protein that contains group-reactive determinants and closely resembles the GroEL homolog (65 kDa) of *Mycobacterium tuberculosis* and *Coxiella burnetii* (35, 42, 49). This family of heat shock proteins is thought to play a role in the response of bacteria to environmental stress (52) and perhaps to facilitate the folding and assembly of oligomeric proteins (17). The 65-kDa antigen of *M. tuberculosis* is an immunodominant protein that is recognized by 20% of the mycobacterium-reactive CD4<sup>+</sup> T lymphocytes in mice (51). The 110-kDa *R. tsutsugamushi* antigen is a less abundant protein which contains both group-specific (31) and strain-specific (29) epitopes.

The reason for the lack of T-cell response to the Sta56, Sta58, and Sta110 antigens, all of which induce strong humoral responses, is unclear; however, these polypeptides are expressed at considerably lower levels in recombinant *E. coli* lysates than in *R. tsutsugamushi* antigen preparations (30, 31), so the lack of response may reflect insufficient antigen concentration. In an effort to concentrate these antigens and possibly remove inhibitory proteins, the Sta58, Sta56, and Sta110 proteins were separated by SDS-PAGE and eluted. Despite successful use of this technique with Sta22 (18), no augmentation of the proliferative response was detected.

Another explanation for the lack of response to Sta56, Sta58, and Sta110 is the absence of T-cell epitopes recog-

nized by C3H/HeJ T cells on these antigens. Results obtained from studies of mycobacteria and *Francisella tularensis* indicate that T-cell-reactive proteins contain a limited number of T-cell determinants (16, 32, 38, 39, 46). For instance, 5 of 10 primed individuals failed to respond to any of a series of overlapping peptides encompassing the 17-kDa lipoprotein derived from *F. tularensis* (46). Additionally, a 40-kDa membrane protein of *F. tularensis* was found to stimulate only 7 of 11 naturally infected persons (44). Similar results have been noted with *M. tuberculosis* (10, 48). Since we are examining the response to only one murine haplotype, recognition of two (Sta22 and Sta47) of five antigens may not be unreasonable. A proliferative response to native eluted fractions that overlap the 110-, 58-, and 56-kDa *R. tsutsugamushi* antigens was seen, although the T-cell proliferative responses observed may be the result of additional *R. tsutsugamushi* antigens present in these fractions but not recognized by the antiserum.

Sequence analysis of pRTS47, the recombinant clone encoding Sta47, revealed the presence of a 47-kDa open reading frame as well as open reading frames potentially encoding proteins of 30, 18, and 13 kDa. SDS-PAGE and elution of Sta47 demonstrated the stimulatory capacities of the region containing the 47-kDa antigen as well as the regions containing the 18- and 13-kDa antigens or small breakdown products of the Sta47 protein. The deduced amino acid sequence of the Sta47 protein was used to identify amphipathic regions, since T-cell antigenic sites tend to be structures with hydrophobic and hydrophilic residues on opposite faces (8). Eight Sta47 synthetic peptides with high amphipathic scores were produced and examined for the ability to stimulate the C3H/HeJ T cell line. One peptide, pepSta47(81-100) (Asp-Gly-Lys-Arg-Val-Ile-Val-Asn-Ala-Phe-Ala-Lys-Phe-Lys-Ile-Ile-Asp-Pro-Ile-Thr), was capable of eliciting a proliferative response in the polyclonal C3H/HeJ T-cell line more than 10 times greater than that elicited by an irrelevant malarial peptide of identical size. PepSta47(91-110), which overlaps pepSta47(81-100) by 10 amino acids, did not stimulate the T-cell line. In addition to the amphipathic predictive algorithm (24) for T-cell antigenic determinants, Rothbard and Taylor, by analysis of primary structure, have identified two motifs common to T-cell determinants (34). Interestingly, when the peptide 81-100 sequence is examined visually for these antigenic determinant motifs, two sequences are found. One region, Asn-Ala-Phe-Ala-Lys, encompasses amino acids 88 to 92 and therefore would not be completely present in peptide 91-110. If this were the core of the antigenic determinant recognized by the C3H/HeJ T-cell line, the lack of response with overlapping peptide 91-110 could be easily explained, since residues 88 to 90 and possibly additional stabilizing residues would be lacking in peptide 91-100. An additional region in peptide 81-100 conforms to the motif of Rothbard and Taylor (34) and is found at amino acids 94 to 97 (Lys-Ile-Ile-Asp). Since this sequence and three flanking amino acids would be present in peptide 91-110, it is less likely that this is the antigenic determinant recognized by C3H/HeJ (*H-2<sup>k</sup>*) T cells. This sequence and others present within the nonstimulatory peptides tested could be of importance, however, in binding to other haplotypes. Thus, peptide 81-100, obtained from Sta47, contains a T-cell epitope recognized by a polyclonal C3H/HeJ *R. tsutsugamushi*-responsive T-cell line.

The identification of open reading frames potentially encoding *R. tsutsugamushi* proteins of 18 and 13 kDa in combination with the strong T-cell response to both native



and recombinant eluted antigens in the <18-kDa range demonstrates that these antigens warrant more-detailed analysis and characterization. The recognition of the 47-kDa *R. tsutsugamushi* antigen by a T-cell line derived from animals immunized with a native antigen preparation containing a myriad of potential antigenic determinants implicates this antigen as one of importance in future protection studies and vaccine development.

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