Characterization of CD8+ Effector T Cell Responses in Volunteers Immunized with Salmonella enterica Serovar Typhi Strain Ty21a Typhoid Vaccine

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Characterization of CD8+ Effector T Cell Responses in Volunteers Immunized with *Salmonella enterica* Serovar Typhi Strain Ty21a Typhoid Vaccine

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*Salmonella enterica* serovar Typhi (*S. typhi*) strain Ty21a remains the only licensed attenuated typhoid vaccine. Despite years of research, the identity of the protective immunological mechanisms elicited by immunization with the Ty21a typhoid vaccine remains elusive. The present study was designed to characterize effector T cell responses in volunteers immunized with *S. typhi* strain Ty21a typhoid vaccine. We determined whether immunization with Ty21a induced specific CTL able to lyse *S. typhi*-infected cells and secrete IFN-γ, a key effector molecule against intracellular pathogens. We measured the functional activity of these CTL by a 51Cr-release assay using 8-day restimulated PBMC from Ty21a vaccinees as effector cells and *S. Typhi*-infected autologous PHA-activated PBMC as target cells. Most vaccinees exhibited consistently increased CD8-mediated lysis of targets by postimmunization PBMC when compared with preimmunization levels. We also developed an IFN-γ ELISPOT assay to quantify the frequency of IFN-γ secretory cells (SFC) in PBMC from Ty21a vaccinees using an ex vivo system. Significant increases in the frequency of IFN-γ SFC following immunization (mean ± SD, 393 ± 172; range 185–548 SFC/10⁶ PBMC; *p* = 0.010), as compared with preimmunization levels, were observed. IFN-γ was secreted predominantly by CD8⁺ T cells. A strong correlation was recorded between the cytolytic activity of CTL lines and the frequency of IFN-γ SFC (*r*² = 0.910, *p* < 0.001). In conclusion, this work constitutes the first evidence that immunization of volunteers with Ty21a elicits specific CD8⁺ CTL and provides an estimate of the frequency of CD8⁺ IFN-γ-secreting cells induced by vaccination. *The Journal of Immunology*, 2002, 169: 2196–2203.

*S. enterica* serovar Typhi (*S. typhi*), the causative agent of typhoid fever, is a human-restricted intracellular Gram-negative bacterium that infects both phagocytic and nonphagocytic cells (1, 2). Worldwide, typhoid fever affects ~16 million individuals annually with 600,000 deaths (3). In Asia and northeast Africa, the appearance of *S. typhi* showing resistance to many antibiotics has become an important public-health problem (4). Therefore, an improved prophylactic vaccine to prevent typhoid fever is urgently needed.

Although much is known regarding the immune responses elicited by *Salmonella typhimurium* in the murine model (1, 5), which results in a typhoid-like disease, little is known about the protective immune responses to *S. typhi* infection in humans. Because of the narrow restriction of *S. typhi* for human hosts, definitive studies in humans are desirable (6). Results from studies in typhoid patients and vaccine trials with attenuated *S. typhi* indicate that Abs appear to be involved in protection against *S. typhi* (2, 6, 7). However, the role of cell-mediated immunity (CMI) in protection from *S. typhi* infection remains unclear. There is considerable evidence that host resistance to many intracellular bacteria such as *Listeria monocytogenes* (8, 9), and *Mycobacteria* (10, 11) is strongly influenced by CMI responses. We have previously demonstrated the presence of specific CTL and IFN-γ production to *S. typhi* Ags in volunteers immunized with attenuated *S. typhi* strain CVD 908 and suggested that these might be important effector mechanisms in resistance to *S. typhi* infection (12, 13).

Despite years of research, there is little information on the protective immunological mechanisms elicited by oral immunization with *S. typhi* strain Ty21a typhoid vaccine (the only licensed attenuated live vaccine) which has been shown to have a variable rate of protection depending on the formulation used and the number and spacing of the doses administered (2). The purpose of this study was to determine whether immunization with Ty21a typhoid vaccine elicited two key T cell-mediated effector mechanisms, i.e., specific CTL able to lyse *S. typhi*-infected targets and production of IFN-γ in response to stimulation with *S. typhi*-infected cells.

**Materials and Methods**

**Subjects**

Five healthy volunteers, between 24 and 41 years of age, recruited from the Baltimore-Washington area and University of Maryland at Baltimore campus (Baltimore, MD), participated in this study. They were immunized with four spaced doses of 2–6 × 10⁹ CFU of Ty21a at an interval of 48 h between doses (14). Healthy volunteers underwent leukapheresis following standard procedures before and between 4 and 37 mo (mean = 16 mo) after ingestion of the vaccine. PBMC were then isolated by density gradient centrifugation and cryopreserved in liquid N₂. Demographic characteristics of volunteers, as well as their HLA haplotypes, are shown in Table I. Volunteer were without any antibiotic treatment and had normal blood counts at the times of leukapheresis. Before the leukapheresis procedures were performed, the purpose of this study was explained to volunteers and they signed informed consents.

**Preparation of target/stimulator cells**

Blasts were obtained by incubating 5–10 × 10⁹ PBMC with 1 μg/ml PHA-L (Sigma-Aldrich, St. Louis, MO) for 24 h in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 100 U/ml penicillin,
In control experiments, inactivated Dr. J. Nataro, Center for Vaccine Development, University of Maryland assays. Sodium chromate (\textsuperscript{51}Cr) (Amersham Pharmacia Biotech, Piscataway, NJ) from PBMC isolated from Ty21a vaccinees following standard procedures. PHA-activated PBMC were then washed three times with RPMI 1640, and cultured in complete RPMI supplemented with 20 IU/ml recombinant human IL-2 (rhIL-2) (Boehringer Mannheim, Mannheim, Germany) for 5–6 days.

EBV-transformed lymphoblastoid B cell lines (B-LCL) were established from PBMC isolated from Ty21a vaccinees following standard procedures (13, 15). B-LCL were maintained in culture in complete RPMI or cryopreserved until used in the experiments.

**Infection of target/stimulator cells by S. typhi**

PHA-stimulated PBMC (henceforth called “blasts”), were incubated in RPMI (without antibiotics) for 3 h at 37°C, 5% CO\textsubscript{2}, in the absence or presence of wild-type S. typhi strain ISP1820 (wt S. Typhi) (obtained from Dr. J. Nataro, Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, MD) at a different multiplicity of infection (MOI). In control experiments, inactivated S. Typhi (licensed heat-inactivated phenol-preserved typhoid vaccine, typhoid vaccine USP; Wyeth-Ayerst Pharmaceuticals, Marietta, PA) was added at a MOI of 10:1. After exposure to S. Typhi, cells were washed and incubated overnight at 37°C, 5% CO\textsubscript{2}, in complete RPMI containing 20 IU/ml of rhIL-2. The following day, cells were gamma-irradiated (4000 rad) and used as stimulators to expand effector cells for CTL assays or in ELISPOT assays. Alternatively, infected and uninfected blasts or B-LCL were labeled with 200 \textmu Ci of sodium chromate \textsuperscript{51}Cr (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 h at 37°C, 5% CO\textsubscript{2}, washed three times, and used as targets in CTL assays.

**Analysis of intracellular and surface S. typhi Ags**

Intracellular staining was performed following standard techniques (16). Briefly, blasts infected with S. typhi, or not infected, were removed from culture after 3 h of incubation, washed with PBS and fixed with PBS/4% formaldehyde (Polysciences, Warrington, PA). After 30 min at room temperature, cells were washed with PBS and incubated for 10 min with 150 \textmu M PBS/1% BSA/0.5% saponin (permeabilization buffer; Sigma-Aldrich). Optimal concentrations of a FITC-labeled polyclonal Ab recognizing *Salmonella* common structural Ags (CSA-1; Kirkegaard & Perry Laboratories, Gaithersburg, MD) or a mouse IgG1-FITC isotype control (BD Immunocytometry Systems) were then added and the mixture was incubated for 30 min at room temperature. Anti-CSA-1 is an Ab broadly reactive to *Salmonella* that was purified by affinity chromatography from a pool of serum from goats immunized sequentially with different *Salmonella* strains, including *S. typhi*. Cells were then washed twice with permeabilization buffer, and once with PBS. Samples were analyzed with an Epics Elite ESP flow cytometer/cell sorter system (Beckman Coulter, Miami, FL).

*S. typhi* Ag expression was also determined on infected and not infected CD\textsuperscript{4} or CD\textsuperscript{8} T cells, NK, B cells, and macrophages within the blast populations by multicolor surface staining after 16 h of incubation. To this end, blasts were stained with CSA-1-FITC (Kirkegaard & Perry Laboratories) or mAbs to CD3-FITC (clone UCHT1; Beckman Coulter), CD4-PE-Texas Red (ECD) (clone SFC127F4011; Beckman Coulter), CD8-PerCP (clone SK1; BD Immunocytometry Systems), CD14-allophycocyanin (ALPC) (clone MoP9; BD Immunocytometry Systems), CD19-PerCP (clone SJ25-C1; BD Immunocytometry Systems), CD56-PE (clone B159; BD Immunocytometry Systems) in various combinations and analyzed using an Epics Elite ESP flow cytometer/cell sorter system.

**Preparation of effector cells**

For cytotoxic assays, we used as effector cells both ex vivo- and in vitro-expanded PBMC from immunized volunteers. In vitro-expanded effector cells were obtained using a modification of a previously described technique (17). Briefly, PBMC were cocultured with stimulator cells at an effector to stimulator cell ratio of 7:1 in complete RPMI supplemented with 60 IU/ml of rhIL-2 for 7–8 days. Stimulator cells consisted of autologous blasts infected with S. typhi and gamma-irradiated (4000 rad) as described above. Aliquots of effector cells were used for phenotypic analysis by flow cytometry. Effector cells were stained with mAbs to CD3, CD4, CD8, CD56, TCR\textgamma-\textdelta-FTC (clone WT31; BD Immunocytometry Systems), and TCR\textgamma-\textdelta-ALPC (clone B1.1; BD Immunocytometry Systems) conjugated to the appropriate fluorochromes and analyzed by five-color flow cytometry.

**For IFN-\gamma ELISPOT assays, PBMC from immunized volunteers were used ex vivo as effector cells. In some experiments, PBMC were fractionated into CD4-, CD8-, or NK-depleted subsets using anti-CD4 and CD8 immunomagnetic beads (Dynal Biotech, Great Neck, NY) or anti-NK microbeads (Miltenyi Biotec, Auburn, CA). Alternatively, purified cell populations were obtained by flow cytometric cell sorting following staining with the appropriate mAbs. Cell populations were >90% pure as determined by flow cytometric analysis.**

### Table 1. Summary data on the characteristics of Ty21a vaccinees participating in this study

<table>
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<td>DRB1*0301, 11041</td>
<td>DRB1*0701</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Numbers represent the age of volunteers before immunization.

\textsuperscript{b} Performed by PCR-sequence-specific oligonucleotide probes.

100 \mu g/ml streptomycin, 50 \mu g/ml gentamicin, 2 mM L-glutamine, 2.5 mM sodium pyruvate, 10 mM HEPES buffer, and 10% heat-inactivated FBS (complete RPMI). PHA-activated PBMC were then washed three times with RPMI 1640, and cultured in complete RPMI supplemented with 20 IU/ml recombinant human IL-2 (rhIL-2) (Boehringer Mannheim, Mannheim, Germany) for 5–6 days.

**Compartment**

Cytotoxicity was determined by a 4-hr \textsuperscript{51}Cr-release assay as previously described (17). Spontaneous release was determined from wells containing medium alone; maximum release was determined from wells to which 2% Triton-X (Sigma-Aldrich) was added. All cultures were set up in quadruplicate. Lysis (%) was calculated as follows: ((experimental release – spontaneous release)/(maximum release – spontaneous release) \times 100). Specific cytotoxicity mediated by effector cells was calculated by subtracting the lysis of uninfected targets from the lysis of *S. typhi*-infected targets. The cut-off for positive responses in CTL assays was defined as >10% specific lysis above the mean specific lysis of effector PBMC when cultured with not-infected target cells as previously described (18).

**Competitive inhibition studies were conducted by measuring the specific lysis of labeled target cells by a fixed number of effector cells in the presence of varying numbers of unlabeled target cells as previously described (17).**

**Analysis of intracellular levels of IFN-\gamma**

Identification of the effector cell populations secreting IFN-\gamma following exposure to *S. typhi*-infected targets was performed by multicolor flow cytometry. To exclude from analysis target cells that might secrete IFN-\gamma, uninfected or *S. typhi*-infected target cells were labeled with a mAb to CD45-ALPC (a leukocyte common surface Ag marker; clone IM2473; Beckman Coulter). CD45-stained target cells were then cocultured with effector cells at an E:T cell ratio of 1:7. Effector cells cultured without target cells or with anti-CD3/CD28 beads (0.6 \mu g/ml; Dynal Biotech) were used as negative and positive controls, respectively. After 16 h of incubation at 37°C, cytokine secretion was blocked by the addition of brentelim A (Sigma-Aldrich) at a final concentration of 10 \mu g/ml for 5–6 h. Cells were then harvested, washed with PBS, and surface-stained with CD56-FITC,
CD4-ECD, and CD8-PerCP mAbs. After washing, cells were stained for intracellular IFN-γ content using a protocol similar to the one described above for the analysis of intracellular S. typhi Ags. In this case, using an anti-IFN-γ polyclonal Ab (clone 4S.B3; BD ImmunoCytometry Systems). In these experiments, stained cells were analyzed by flow cytometry by first gating on the CD45- cell population to exclude target cells, followed by electronic gating on the CD3 CD56+, CD3 CD44+, or CD3 CD8+ populations for the determination of IFN-γ+ cells in each of them. In other experiments, effectors were stained with a combination of CD-3-FTC, CD65-PE, CD4-PerCP-Cy5.5 (clone RPA-T8; Becton Dickin-
sion), and IFN-γ ALP mAbs. In these experiments, cells were analyzed by sequential gating on CD3+, CD8+, CD4+, and CD56+ and the results were reported as % IFN-γ+ cells in this population.

IFN-γ ELISPOT assay

The frequency of IFN-γ-secreting cells was quantified by using a modified IFN-γ ELISPOT assay. Anti-human IFN-γ mAb (5 μg/ml, clone 2G1; Endogen, Woburn, MA) was diluted in coating buffer (PBS/0.4 M NaOH, 2 mM EDTA), and 100 μl/well were added to dry MultiScreen-HA filtration plates (MAHA S4510; Millipore, Bedford, MA). After overnight in-
cubation at 4°C, the wells were washed with wash buffer (PBS/
0.05%;Tweent 20) and unoccupied sites were blocked with 200 μl/well PBS/5% BSA for 2 h at room temperature. After washing, 200 μl/well complete medium was added and incubated at room temperature. After 1 h, the complete RPMI was discarded and stimulator-effector cells at a 1:7 ratio were seeded in 200 μl/well and incubated in a humidified 37°C, 5% CO2 incubator. Effector cells cultured without stimulator cells or with CD3/CD28 beads (0.6 μM; Dynal Biotech), were used as negative and positive controls, respectively. Target cells uninfected or infected with S. typhi without effector cells were also used as controls. After 16 h of un-
disturbed incubation, wells were washed and incubated for 2 h at room temperature with 100 μl/well of biotin-labeled anti-human IFN-γ mAb (clone B13.3.5; Endogen) (2 μg/ml in PBS/1% BSA, ELISPOT buffer). After washing, 100 μl/well of avidin-peroxidase (Sigma-Aldrich), diluted 1/400 in ELISPOT buffer, were added and incubated at room temperature for 2 h. Wells were then washed and 50 μl/well of the detection substrate TrueBlue (Kirkegaard & Perry Laboratories) were added. After 15 min., wells were washed with distilled water and allowed to dry. Spots were enumerated using a stereomicroscope. Net frequencies of IFN-γ-spot-form-
ing cells (SFC) were calculated by using the following formula: ([number of SFC in effector cell populations incubated with S. typhi-infected targets] − [number of SFC in effector cell populations incubated with un-
infected targets + number of SFC in cultures containing S. typhi-infected target cell populations alone]). The cut-off for positive responses in IFN-γ ELISPOT assays (175 SFC/106 PBMC) was established by calculating the average frequency of IFN-γ-producing cells/106 PBMC when cultured with un-infected target cells + 3 SE.

Statistical analysis

Comparisons between the expression of S. typhi Ags in the different stimulator/target cell subpopulations were performed by one-way ANOVA tests. Comparisons of cytotoxicity levels and IFN-γ produc-
tion by ELISPOT before and after immunization were performed by Student’s t tests. Linear regression analysis was used to correlate the results obtained by CTL and IFN-γ ELISPOT assays. All tests were performed using SigmaStat software (version 2.03; SSPS, Chicago, IL). Values of p < 0.05 were considered significant.

Results

Expression of S. typhi Ags in PHA-stimulated PBMC

We have previously demonstrated the presence of specific CTL responses in volunteers immunized with attenuated S. typhi strain CVD 908 and suggested that this might be an important effector mechanism in resistance to S. typhi infection (13). To determine whether immunization with the licensed typhoid vaccine attenu-
ated strain Ty21a also elicits the appearance in circulation of CTL able to lyse infected targets, we modified the CTL assay previously described (13) by using autologous S. typhi-infected blasts as targets instead of B-LCL. As a preliminary step in the generation of appropriate target cells, we evaluated whether wt S. typhi was able to infect blasts and express Ags on their cell membranes. To this end, blasts were incubated with medium alone or with wt S. typhi for 3 h at different MOI. After this incubation, cells were har-

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vested, washed three times with RPMI containing gentamicin (100 μg/ml) to kill extracellular bacteria, and the presence of intracel-
lar S. typhi Ags was examined immediately using the intracel-
ular staining procedure described in Materials and Methods. Alter-
atively, cells were incubated for an additional 13 h in complete RPMI containing gentamicin (100 μg/ml) before being examined for surface expression of S. typhi Ags. We observed that after 3 h of incubation ~90% of blasts exposed to S. typhi at various MOI expressed S. typhi Ags intracellularly (Fig. 1, B–D). In contrast, no significant expression of S. typhi Ags was observed in the cyto-
plasm of uninfected cells (mean = 1.9%, Fig. 1A). Similarly, >90% of infected cells were found to express S. typhi Ags on their cell membrane following 16 h of incubation in the presence of gentamicin (Fig. 1, F–H), whereas only a small proportion of un-
infected cells (Fig. 1E) or cells exposed to inactivated S. typhi (Fig. 1I) stained positively (range 1–6%) in several repeat experiments. These results suggest that expression of S. typhi Ags on the cell surface is dependent on bacterial cell invasion. The observed kinet-
ics of expression of bacterial Ags is similar to that reported by us (13) and others (19) using similar culture conditions.

To determine whether a particular T cell population was prefer-
entially infected and expressed S. typhi Ags, infected blasts were stained with mAbs to CD3, CD4, and CD8 in addition to the anti-S. typhi CSA-1 polyclonal Ab. As can be observed in Fig. 1, I–K, the vast majority of CD3+ CD4+, as well as CD3+ CD8+, expressed S. typhi Ags on the cell membrane. In the experiment shown, the mean percentages of S. typhi-expressing cells among the different PBMC subpopulations were 79% of CD3+ T cells, 86% of CD4+ T cells, and 82% of CD8+ T cells. No statistical differences were observed among CD4+ and CD8+ cell populations expressing S. typhi Ags following S. typhi infection in the various repeat ex-
periments with cells from three different donors (p = 0.472). Other populations, such as NK cells, B cells, and macrophages, were present in very small numbers in blast cell populations, making it difficult to establish whether these cell populations express S. typhi Ags on their cell membrane following S. typhi infection. Taken together, these results demonstrated the ability of S. typhi to effi-
ciently infect a large percentage of CD3+CD4+ and CD3+CD8+ blasts. Based on these findings, a MOI of 20:1 and an incubation period of 16 h were chosen for the infection of blasts in subsequent studies.

Induction of CTL activity by immunization with Ty21a typhoid vaccine

To determine whether immunization with the Ty21a typhoid vaccine elicits the appearance of effector CTL able to lyse S. typhi Ag-expressing blasts, PBMC from Ty21a typhoid vaccinees were used ex vivo (i.e., immediately after isolation) as effectors in 51Cr-
release cytotoxicity assays. These experiments showed that ex vivo PBMC from four volunteers were unable to lyse autologous blasts infected with S. typhi (data not shown). We next evaluated the ability of PBMC from these volunteers to lyse S. typhi Ag-expressing blasts following an 8-day in vitro expansion with irradi-
ated autologous blasts infected with S. typhi at a PBMC-stimulator ratio of 7:1. This ratio was selected based on preliminary experi-
m ents showing that it generated the highest numbers of effectors with the highest viability, as indicated by their ability to exclude trypan blue (data not shown). As shown in Fig. 2A, in experiments using preimmunization PBMC as effector cells, low levels of spe-
cific CTL activity were observed in three of four volunteers at the 10:1 and 3:1 E:T ratios. Significant increases in CTL activity fol-
lowing immunization were observed in three of four volunteers (Fig. 2A). Although small increases in CTL activity were observed in the remaining volunteer (MP), these increases did not reach
statistical significance (Fig. 2A). No specific cytotoxic activity was observed when uninfected autologous blasts or blasts exposed to inactivated S. typhi were used as targets in these assays (Fig. 2B), indicating that infection is necessary to enable killing of targets by effector CTL.

In two Ty21a-immunized volunteers from whom sufficient cells were available, similar CTL results were observed with PBMC expanded in vitro with S. typhi-infected autologous blast and evaluated against S. typhi-infected autologous B-LCL or blasts (data not shown). These results are consistent with our previous observations that oral immunization of volunteers with attenuated S. typhi strain CVD 908 induces specific cytotoxicity against S. typhi-infected autologous B-LCL (13).

The specificity of CTL effector cells was further investigated by competitive inhibition assays using standard techniques (17). As shown in Fig. 3, lysis of 51Cr-labeled autologous S. typhi-infected blasts was inhibited in a dose-dependent fashion by unlabeled autologous S. typhi-infected blasts. In contrast, no inhibition was observed when unlabeled autologous uninfected blasts were added to the cultures.

Characterization of the effector CTL population

As a first step to identify the effector cell population responsible for CTL activity, PBMC obtained from Ty21a-immunized volunteers following an 8-day in vitro expansion with S. typhi-infected autologous blasts were used in CTL assays unfractionated or following depletion of NK cells using anti-CD56-coated magnetic beads. Flow cytometric analysis of the depleted cell populations consistently showed that they contained <1.5% CD56+ NK cells. Depletion of NK cells did not affect the ability of in vitro-expanded PBMC from these volunteers to lyse autologous S. typhi-infected blasts, indicating that NK cells are not responsible for the observed CTL activity in unfractionated PBMC populations (data not shown). Of note, depletion of NK cells decreased to a considerable extent the observed background cytotoxicity (10–25% at 10:1 E:T ratio).

To identify the T cell subpopulation responsible for the observed lysis of autologous S. typhi-infected targets, CD3+CD8+ CD56− or CD3+CD8+CD56+ cell populations were isolated from PBMC obtained from Ty21a-immunized volunteers by positive flow cytometric sorting. Purity of the sorted cell populations was routinely >90% CD3+CD8− for positively sorted populations, as determined by flow cytometric analysis. Results demonstrated that CTL activity against autologous S. typhi-infected blasts was mediated by CD3+CD8+CD56− T cells, while no significant activity was observed in cultures containing CD3+CD8+CD56+ cells (Fig. 4). To evaluate whether the CD3+CD8+CD56− CTL effector populations express TCRβ or TCRγδ, expanded PBMC were stained with mAbs to CD3, CD4, CD8, TCRβ, and TCRγδ and analyzed by five-color flow cytometry. Results indicated that virtually all CD3+CD8+ express TCRβ while <1% expressed TCRγδ (data not shown). Taken together, these findings demonstrate that CD3+CD8+CD56− TCRβ+ CTL effectors isolated from volunteers immunized with S. typhi strain Ty21a are responsible for the killing of autologous S. typhi-infected targets.

Induction of IFN-γ-secreting effector T cells

We next examined whether immunization with Ty21a elicits the appearance of effector cells able to secrete IFN-γ in response to stimulation with S. typhi-infected blasts, as well as the frequency of these effector cells. To this end, we developed an ex vivo IFN-γ ELISPOT assay using PBMC from immunized volunteers as effectors and autologous blasts infected with wt S. typhi as stimulators. Significant increases in the net frequency of IFN-γ SFC were observed in four of five volunteers following immunization (Fig. 5). Increases in the net frequency of IFN-γ SFC following immunization as compared with preimmunization levels ranged from 185–548 SFC/10⁶ PBMC (mean ± SD, 393 ± 172).

In control cultures, the presence of IFN-γ-producing cells in effector PBMC incubated with uninfected blasts (mean ± SD, 220 ± 119; range 0–403 SFC/10⁶ PBMC) was more frequent than when effectors were incubated in the presence of medium alone.
or infected with blasts uninfected (F autologous target cells (cold targets). The cold targets were autologous ratio of 40:1 in the absence or presence of varying numbers of unlabeled

FIGURE 3. Analysis of CTL activity by competitive inhibition assay. PBMC (mean/H11006 of IFN- (virtually none detected; data not shown). In positive control wells 51 Cr-release assays.

FIGURE 2. Ability of PBMC effectors to lyse S. typhi-infected autologous target cells. A, The ability of 8-day restimulated PBMC obtained pre- or postimmunization to lyse autologous blast targets infected with live S. typhi as described in Materials and Methods. *, p < 0.05 and **, p = 0.10 by Student's t test. B, The ability of PBMC obtained from volunteers postimmunization to lyse targets incubated with medium, inactivated, or live S. typhi. Bars represent the mean percent-specific lysis observed for each volunteer at the indicated E:T cell ratios. Error bars show the SD of quadruplicate cultures. The dashed line represents the cut-off for positive 51Cr-release assays.

(virtually none detected; data not shown). In positive control wells (effector PBMC incubated with CD3/CD28 beads), the frequency of IFN-γ-producing cells ranged from 2,693 to 3,860 SFC/10^6 PBMC (mean ± SD, 3182 ± 451). In three of the volunteers for whom sufficient cells were available, increased numbers of SFC/10^6 PBMC following immunization in response to S. typhi-infected blasts were observed when assayed in three independent experiments (data not shown). We also evaluated in these vol-

FIGURE 4. Specific cytotoxicity of S. typhi-infected blasts is mediated by CD8^+ effector T cells. Unfractionated PBMC, CD3^+CD8^+CD56^- or CD3^+CD8^-CD56^+ sorted cell populations were used as effectors against S. typhi-infected autologous blast targets. Bars represent the mean percent-specific cytotoxicity observed for each target at an E:T cell ratio of 10:1. Error bars show the SD of triplicate cultures. The dashed line represents the cut-off for positive 51Cr-release assays.

Net frequency of IFN-γ-producing cells before and after immunization in the presence of autologous blasts infected with S. typhi. Net frequencies of IFN-γ SFC were assessed by an IFN-γ ELISPOT assay using ex vivo PBMC from immunized volunteers as effectors and autologous blasts infected with wt S. typhi as stimulators. Net frequencies of IFN-γ SFC were calculated as described in Materials and Methods. The dashed line represents the cut-off for positive ELISPOT assays determined as described in Materials and Methods. *, p < 0.05 by Student's t test.

(only) or infected with S. typhi (•). These results are representative of one of two experiments with cells from different donors.

FIGURE 5. Frequency of IFN-γ-producing cells before and after immunization in the presence of autologous blasts infected with S. typhi. Net frequencies of IFN-γ SFC were assessed by an IFN-γ ELISPOT assay using ex vivo PBMC from immunized volunteers as effectors and autologous blasts infected with wt S. typhi as stimulators. Net frequencies of IFN-γ SFC were calculated as described in Materials and Methods. The dashed line represents the cut-off for positive ELISPOT assays determined as described in Materials and Methods. *, p < 0.05 by Student's t test.

Characterization of the IFN-γ-secreting effector cell population

We next investigated which cell populations in PBMC from immunized volunteers secrete IFN-γ when stimulated with S. typhi-infected autologous blasts. To this end, PBMC were negatively depleted of CD8^+, CD4^+, or NK cells using immunomagnetic beads. Flow cytometric analysis of the depleted cell populations indicated that virtually all NK and CD4^+ cells and ~80% of the CD8^+ cells present in PBMC populations were removed by this procedure (data not shown). Depleted cell populations were stimulated with autologous blasts infected or not with S. typhi. Results indicate that CD8 depletion abrogated the presence of IFN-γ-secreting cells in response to S. typhi-infected autologous blasts, whereas depletion of NK cells did not significantly alter the number of IFN-γ-secreting cells observed in unfractionated PBMC (Fig. 6). In contrast, CD4-depleted populations exhibited increased numbers of IFN-γ-secreting SFC, likely the result of increased proportions of CD8^+ T cells in this population. These results suggest that CD8^+ T lymphocytes are the cells within the PBMC population primarily responsible for IFN-γ secretion in response to stimulation with autologous S. typhi-infected cells.

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To confirm that CD8+ T lymphocytes are the cells that secrete IFN-γ in response to stimulation with autologous S. typhi Ag-expressing targets, PBMC from immunized volunteers were cocultured with autologous blasts infected or not with S. typhi. After 16 h (the same time point at which the ELISPOT assay is assessed), the intracellular levels of IFN-γ in the various cell populations were determined by flow cytometry. Preliminary experiments in which CD45 was used to exclude targets cells from analysis demonstrated that <2% of the cells remain CD45+ within the “live” lymphocyte gate (as defined in forward vs side light scatter cytograms) following the overnight stimulation with S. typhi-infected cells. Thus, CD45 staining was not used in subsequent experiments. Costaining for intracellular IFN-γ and cell surface markers demonstrated that CD3+CD4–CD8+CD56– T cells rather than CD3+CD4+CD8–CD56– T cells or NK cells were the dominant sources of IFN-γ in postimmunization effector PBMC populations. Increases of 2- to 5-fold in the frequency of CD3+CD8–CD4+ CD56– cells expressing IFN-γ were observed in populations isolated from volunteers following immunization as compared with preimmunization levels (Fig. 7). In contrast, CD4+CD8+CD56– or CD4+CD8–CD56– cell populations expressing IFN-γ showed a variation of <2-fold (data not shown). Of note, increased numbers of CD3+CD8–CD4+CD56– IFN-γ+ effector cells (3.3%) were observed in volunteer TS (Fig. 7). No increases in IFN-γ production were observed when effectors were incubated in the presence of stimulators exposed to inactivated S. typhi (Fig. 7) indicating that infection is required to trigger IFN-γ production by effectors cells.

**Correlation of CTL and IFN-γ-secreting effector T cell populations in Ty21a vaccinees**

To evaluate whether the frequency of cells that secrete IFN-γ in response to stimulation with autologous S. typhi-infected cells correlates with CTL activity, we compared the results obtained by ELISPOT with those obtained by 51Cr-release assays. A positive correlation was found between the frequency of IFN-γ-secreting cells and the cytotoxic activity of CTL effectors at E:T ratios of 10:1 (r = 0.954, p < 0.001) or at E:T ratios of 3:1 (r = 0.892, p = 0.003; Fig. 8). When we compared the cytolytic activity at an E:T ratio of 10:1 with the frequency of IFN-γ-secreting cells, as measured by intracellular IFN-γ staining (r = 0.751, p = 0.085), or the frequencies of IFN-γ-secreting cells measured by ELISPOT with those measured by intracellular IFN-γ staining (r = 0.755, p = 0.083), we found trends rather than statistical significant correlations, likely because of the small number of volunteers studied.

**Discussion**

In this study, we described an in vitro model using S. typhi-expressing autologous blasts to investigate CMI in volunteers immunized with S. typhi strain Ty21a. Our data showed that oral immunization with attenuated vaccine strain Ty21a induced potent CTL responses as well as IFN-γ production by CD3+CD8+ T cells in volunteers of different HLA-class I haplotypes. Moreover, our data suggest that the Ty21a vaccine can induce cellular immune responses lasting for at least 2 years after immunization (volunteers MR and SD, Table I).

**FIGURE 6.** Cellular sources of IFN-γ-producing cells. PBMC from a Ty21a vaccinee were depleted of CD8+ and CD4+ cells by negative selection using immunomagnetic beads and stimulated with autologous blasts infected or not with S. typhi. After 16 h, IFN-γ SFC were detected using a modified ELISPOT assay. Net frequencies of IFN-γ SFC were calculated as described in Materials and Methods. Error bars show the SD of triplicates cultures. These results are representative of those observed with two different donors evaluated in two independent experiments.

**FIGURE 7.** Detection of intracellular IFN-γ levels in response to S. typhi Ags in CD3+CD8–CD4+CD56– cells. PBMC from immunized volunteers were stimulated with targets cells incubated with medium, inactivated, or live S. typhi for 16 h, stained for CD3, CD8, CD4, and CD56 surface markers and for intracellular IFN-γ as described in Materials and Methods. Data are expressed as the percentage of CD3+CD8–CD4+CD56– cells expressing IFN-γ. Similar results were obtained with an additional vaccinee (RH).

**FIGURE 8.** Correlation between the frequency of IFN-γ SFC detected by ELISPOT assays and cytolytic activity of CTL effectors at E:T ratios of 10:1 (●; long dashed line) (r = 0.954, p < 0.001) and 3:1 (○; short dashed line) (r = 0.892, p = 0.003) detected by 51Cr-release assay. Pre- and postimmunization PBMC from volunteers RH, TS, MR, and MP were used for this analysis.
The efficiency of the immune system to control infection by intracellular pathogens depends to a large extent on the ability of activated effector cells to interact with infected host cells presenting antigenic peptides in the context of HLA class I or II molecules. To investigate the role of CTL in S. typhi infection, we explored the use of S. typhi-infected autologous blasts as APC and as targets. Their use affords a more “physiological target cell population” because they are primary cells and not cell lines like B-LCL. Moreover, because autologous blasts are infected by wt S. typhi, a significant feature of our system is its capacity to express multiple epitopes of S. typhi in the context of various HLA molecules, thereby allowing CTL of different specificities to recognize infected cells. By using a MOI of 20:1, blasts could be infected with high efficiency, as demonstrated by flow cytometric assessment of intracellular and surface S. typhi Ags, and maintained good viability following infection. Another important advantage is its accessibility; blasts can be readily established from PBMC in only 1 wk instead of the ∼4–6 wk required to establish B-LCL. Blasts have been successfully used as target cells in viral systems using human cells (17, 20).

One mechanism by which activated effector cells can contribute to protection is to lyse infected target cells. In this study, PBMC were tested ex vivo against blasts expressing S. typhi Ags. We found that the use of PBMC as effector cells ex vivo did not result in any detectable lysis of S. typhi-infected targets. However, specific expansion of PBMC with S. typhi-infected autologous blasts resulted in strong CTL activity toward S. typhi-infected cells in most volunteers. In these studies, we observed that infection of target cells with live S. typhi is required for lysis by effector CTL. These results extend previous observations with PBMC from volunteers immunized orally with attenuated S. typhi strain CVD 908 which showed that the use of live bacteria is critical to observe CTL activity (13). Of note, low levels of lysis of S. typhi-infected targets by preimmunization CTL were observed at a 10:1 E:T ratio in some volunteers. However, postimmunization levels of CTL activity against S. typhi-infected targets were consistently above this background lysis. This background lysis may be the consequence of cross-reactivity with other Gram-negative bacteria. Previous studies in the murine model have shown that epitopes shared with other Gram-negative bacteria, including S. typhi, can be recognized by a same CTL population (19).

In this report, we showed that CD8+ T cells isolated from Ty21a vaccinees are not only able to lyse S. typhi-infected blasts, but also are potent producers of IFN-γ and demonstrated a significant association between S. typhi-specific CTL and IFN-γ production. These findings strongly support the involvement of cells with CTL activity in IFN-γ production. A close association between the frequency of IFN-γ-secreting cells and CTL activity also has been reported in other systems (31). However, our results do not exclude that different subpopulations of CD8+ T cells might mediate different effector functions, i.e., cytolytic activity or cytokine production, acting in concert and complementing each other.

In summary, this work constitutes the first evidence that immunization of humans with Ty21a typhoid vaccine elicits specific CTL and provides an estimate of the frequency of IFN-γ-secreting cells following oral vaccination. Although our data do not provide a direct indication that CD8+ -mediated CTL and IFN-γ responses play a critical role in protection from typhoid fever, their presence following vaccination with Ty21a suggests that these effector mechanisms might indeed be involved in controlling S. typhi infection. Similarly, we have previously reported that immunization with attenuated strains of S. typhi elicits the appearance in circulation of CD8+, MHC class I-restricted, CTL effector cells capable of killing autologous S. typhi-infected targets, as well as sensitized lymphocytes that proliferate and produce IFN-γ in response to stimulation with S. typhi Ags, suggesting that CMI responses may play a role in limiting the progression of typhoid infection (12, 13). In addition to these CMI responses, it is likely that serum and mucosal Ab responses also contribute to protection. Results from
a field study in volunteers vaccinated with the typhoid Ty21a vac-
cine strain suggested a correlation between increased serum levels of
IgG O Ab and protective efficacy (7). Based on this study,
although serum O Abs are not believed to be the main effector
immunity mechanism in protection to S. typhi infection, increased
levels of IgG to S. typhi LPS have been proposed as a surrogate
marker of protection. Finally, the fact that the licensed parenteral
Vi polysaccharide vaccine elicits serum Vi Abs indicates that al-
most certainly protection with this vaccine is mediated by means
of serum Vi Abs (32).

Direct evidence for the role of CMI in protection from S. typhi
infection might be provided in clinical trials in which CMI re-
A*0201 restricted CD8

References