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Analysis of the Immune Response against Tetanus Toxoid: Enumeration of Specific T Helper Cells by the Elispot Assay

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Abstract

Tetanus toxoid (TT) is an antigen known to induce strong T cell specific immune responses in humans after vaccination. Here we have used the Elispot assay to assess the number of TT-specific Interferon-γ (IFN-γ) secreting T cells present in individuals and monitored the number of TT specific T cells present in the donors for more than two years. In each of the 22 healthy volunteers tested, TT-specific T cells could be detected. Six out of 7 repetitively tested donors showed a remarkably constant number of TT-specific IFN-γ secreting T cells over several months, whereas one donor demonstrated a transient increase during a flu-like infection. Three healthy donors received TT booster-immunizations and showed significant increases in the number of TT-specific IFN-γ secreting T cells which reached peak levels by 4 weeks after vaccination. Depletion of either $CD4^+$ T cells, $CD8^+$ T cells or $CD16^+/CD56^+$ T cells by immunomagnetic separation demonstrated that TT-specific IFN-γ secretion is mediated exclusively by $CD4^+$ T cells. In addition, HLA class-I and -II blocking studies showed that IFN-γ production is performed by HLA class-II restricted cells. Our data show that the Elispot assay can be reliably used to assess the number of TT-specific $CD4^+$ IFN-γ producing cells (i.e. probably T helper cells) and therefore maybe also useful for the assessment of reactions to other helper antigens.

Introduction

Tetanus toxoid (TT) is known to induce a strong and long lasting humoral immune response in humans after vaccination (1). After TT vaccination, uptake of the antigen by antigen-presenting cells (APC) leads to presentation via the MHC molecules (2, 3), followed by induction of clonal T cell response (4–6). It has been demonstrated that TT-specific T cells are mainly $CD4^+$ cells secreting Th1 cytokines such as IFN-γ (7).

Currently, antigen-specific proliferation of T cells is the standard method for the assessment of $CD4^+$ T cell activity (8). Secretion of cytokines is known to be

Abbreviations: TT = tetanus toxoid; IFN-γ = interferon γ; APC = antigen presenting cell; PBMC = peripheral blood mononuclear cell.

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another indicator of specific T cell responses (9) that can either be measured by determination of soluble cytokines in cell culture supernatants or by intracellular cytokine staining, a quantitative method to analyze T cell responses on an individual cell basis (10).

The Elispot assay is another highly sensitive and reproducible method to determine specific T cell responses by quantifying antigen-dependent cytokine secretion at a single cell level. However, most of the studies using the Elispot assay were performed with $CD8^+$ cytotoxic T cells (11, 12). In the present study the Elispot assay was used to analyze TT-specific IFN-γ secretion by peripheral blood mononuclear cells (PBMC) of healthy donors. The Elispot protocol to monitor antigen-specific $CD8^+$ T cells has been established before (11, 13). Here, we analyzed the frequency of TT-specific T cells in healthy donors before and after TT booster immunization. Our data show clear evidence that the IFN-γ-secreting T cell subpopulations specific for TT are $CD4^+$ MHC class II restricted T helper cells.

Materials and Methods

Blood donors and tetanus toxoid immunization

Heparinized blood samples were taken from healthy donors after obtaining informed consent. All donors had been immunized previously against tetanus toxoid (TT) within one to ten years. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Ficoll-Hypaque 1.077 (Pharmacia, Freiburg, Germany). Cells were washed twice with phosphate-buffered saline (PBS, Biochrom, Berlin, Germany). For booster-immunization some volunteers received a single 0.5 ml (75 I. E.) intramuscular dose of aluminum-adsorbed TT (T-IMMUN, Immuno, Heidelberg, Germany). Blood samples were collected before and at defined time points after vaccination as indicated in Figure 3.

Interferon-γ Elispot assay

The assay was adapted from an established protocol to monitor antigen-specific $CD8^+$ cytotoxic T cells (11;13). Concentrations of cells, antibodies and TT antigen as well as incubation times were optimized in pilot experiments (data not shown). 96-well nitrocellulose plates (Millititer, Millipore, Bredford, MA, USA) were coated overnight with 50 µl/well of anti-IFN-γ monoclonal antibody (MAb) (8 µg/ml, in PBS, code-no. 1598-00, Genzyme, Rüsselsheim, Germany) at 4°C. Wells were washed with PBS and blocked with RPMI-1640 containing 10% human AB-blood group-serum for two hours at 37°C. PBMC (1×10^5 cells/well) in a total volume of 200 µl RPMI-1640 containing 10% AB-serum, 2 mM L-Glutamin, 100 U/ml penicillin, streptomycin and 1 mM MEM Sodiumpyruvat (complete cell culture medium) were incubated with 3 µg/ml TT (Calbiochem-Novabiochem, La Jolla, CA, USA; purity tested by SDS-PAGE according to the manufacturer). Each sample was tested in triplicates. Unstimulated PBMC or PBMC stimulated with 1 µg/ml pokeweed mitogen (PWM; Sigma, St. Louis, MO, USA) served as negative and positive controls, respectively. After 20 hours of incubation at 37°C with 5% CO_2, coated plates were washed six times with PBS containing 0.05% Tween 20. The wells were then incubated for 24 hours at 4°C with 100 µl/well biotinylated mouse anti-human IFN-γ MAb (2.5 µg/ml in PBS; clone 4S.B3, Pharmingen, Hamburg; Germany). After washing four times with PBS, 100 µl/well streptavidine-alkaline phosphatase (BioRad, Munich,Germany) diluted 1:1000 in PBS was added to each well and incubated for an additional 2 hours at room temperature. After two more washing steps with PBS, 100 µl/well BCIP/NBT substrate (BioRad) was added for 20–30 min. Color development was stopped by washing the plates with tap water. Plates were then air-dried overnight. Spots were counted using a stereo-

microscope. Mean numbers were calculated by counting spots from triplicate wells and the number of spots from negative controls, wells without TT, were subtracted. In the vast majoritiy of tests negative controls did not exceed two spots/10^5MNC.

Immunomagnetic cell separation

In some experiments $CD4^+$ T cells, $CD8^+$ T cells or $CD16^+/CD56^+$ T cells were depleted from PBMC by two rounds of immunomagnetic separation using a MS^+ selection column according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Thereafter, cells were washed and resuspended in complete cell culture medium. Purity of the unseparated PBMC and the separated fractions were determined by flow cytometry. Phenotype analysis of unseparated PBMC revealed a population of cells containing 30-38% $CD4^+$ cells and 15–19% $CD8^+$ cells. The CD8-depleted cell fraction contained < 1% $CD8^+$ T cells and 42–50% $CD4^+$ T cells, while the CD4-depleted cell fraction contained < 2% $CD4^+$ T cells and 18–30% $CD8^+$ T cells. The sample depleted of $CD8^+$ and $CD16/CD56^+$ cells in an additional experiment contained <1% $CD8^+$ T cells, <2% $CD16^+/CD56^+$ cells and 60% $CD4^+$ cells. Monocytes (CD4 low, $CD14^+$), required for antigen presentation, were still present within the various T cell depleted cell subpopulations.

Blocking studies

Purified anti- HLA-DR (clone B8.12.2, Immunotec, Marseille, France) and mouse anti-human HLA-ABC mAB (clone W6/32, Serotec, Kidlington, Oxford, GB) were used for the blocking studies. Antibodies were used at final concentrations of 1 and 10 μg/ml and added to the cell cultures 30 minutes prior to the addition of TT. The antibody W6/32 was dialyzed before use to make it suitable for cell culture.

Results

Frequency of TT-specific T lymphocytes in healthy immunized donors

The frequency of TT-specific, IFN-γ secreting T cells present in 22 healthy blood donors was established using the Elispot assay (see Fig. 1). All donors had previously obtained active TT immunization within the last one to ten years. Results showed a broad range in the total number of TT-specific T cells present in the different donors. However, we detected the presence of TT-specific IFN-γ releasing cells in each of the 22 donors (Fig. 1). The number of TT-specific T cells present did not clearly correlate with the time interval since the last TT immunization.

In 7 donors, the frequency of IFN-γ releasing TT-specific T cells could be monitored over several months. A reproducible and fairly constant number of TT-specific T cells in the different donors (Fig. 2) could be observed. However, one donor showed a transient increase of TT-reactive IFN-γ secreting T cells. This specific blood sample was taken when the donor suffered an episode of flu-like illness with fever and chills. After recovery, the number of TT-specific T cells present in this donor decreased to baseline.

Kinetics of TT-specific T cell responses in donors after TT vaccination

Three healthy volunteers received TT booster immunization i.m.. The frequency of TT-specific T cells present was determined prior to and following immunization. A significant increase in the number of TT-specific IFN-γ secreting T cells was observed in all

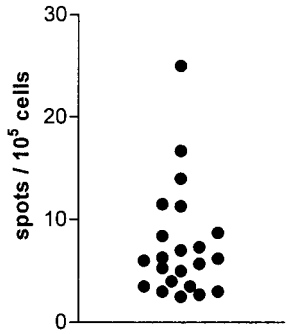

Figure 1. Baseline frequency of TT-specific cells. The number of cells secreting IFN-γ in response to TT in the Elispot assay is shown. PBMC from healthy donors, immunized with TT 1–10 years ago, were cultured in the presence of 3 μg/ml TT for 20 hours. Results are shown as the mean number of spots/1×10^5 PMBC in 22 healthy donors. The numbers of spots from control wells without TT are subtracted.

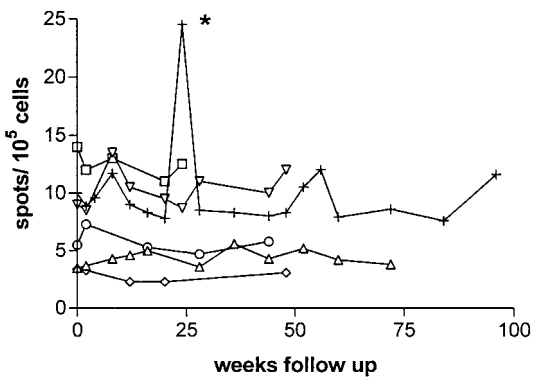

Figure 2. The number of TT-specific cells in seven donors repetitively determined over time is shown. Blood was taken at different time points as indicated. One donor showed an increase in IFN-γ secreting cells during a flu-like infection (*).

three donors. The T cell population reached maximum at week 2 after vaccination in two donors (no earlier time points were taken) and at week 4 in one donor (Fig. 3) and demonstrated a slow decline over a period of several months.

TT-specific IFN-γ secreting T cells are $CD4^+$ helper cells

Two sets of experiments were performed to prove that TT-specific responses are induced by HLA class II-restricted $CD4^+$ T helper cells. Depletion of either $CD4^+$- or $CD8^+$ T cells demonstrated that IFN-γ secreting TT-specific T cells reside within the $CD4^+$ compartment (Fig. 4a). Almost no IFN-γ secreting cells were found within the $CD4^+$ depleted population whereas the number of cells present in CD8-depleted PBMC was slightly higher than those present in the unseparated PBMC. In an additional experi-

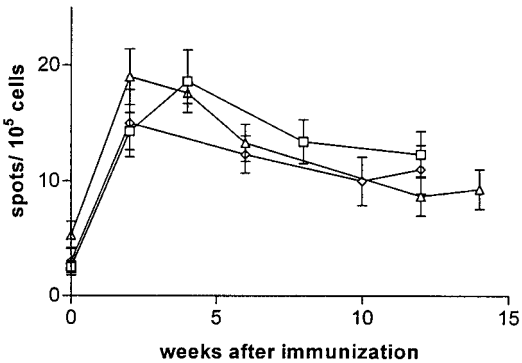

Figure 3. Increase in the frequency of TT-reactive cells post-immunization. TT-specific IFN-γ secreting cells of three volunteers were measured by the Elispot assay before (week 0) and several weeks after booster-immunization with 75 I. U. TT injected i.m. Results are shown as the mean number of spots/ 10^5 PBMC ±SD of triplicate wells.

ment, $CD16^+/CD56^+$ cells were depleted together with the $CD8^+$ cells. Here we observed 15 spots/well in unseparated PBMC, 3 spots/well in $CD4^+$ depleted sample, 23 spots/well in the $CD8^+$ depleted sample and 12 spots/well in the $CD8^+/CD16^+/CD56^+$ depleted cells.

The specific release of IFN-γ induced by TT-reactive T cells could also be inhibited by increasing concentrations of an anti-HLA-DR mAb (B8.12.2). This antibody is known to block 80% of the proliferative response in a mixed lymphocyte culture (15). In control experiments, an anti-HLA-ABC mAb (W6/32) was used. This mAb had previously been shown to block the response of HLA class I restricted $CD8^+$ influenza-specific T cells by 80% in a similar Elispot assay (11). However, W6/32 did not show any inhibition of IFN-γ secretion by TT-specific T cells (Fig. 4b).

Discussion

Using the Elispot assay we could quantify the T cell numbers against a typical T helper cell antigen. TT-specific T cells could be detected in each of the 22 TT preimmunized healthy blood donors. The frequency of TT-specific T cells measured by Elispot assay was in the range of 1:3000–1:30000. Data obtained by others using limiting dilution analysis of IL-2 secreting cells (up to 1/1500) are in accordance with these numbers (4).

Monitoring the frequency of TT-specific T cells for almost 2 years revealed that their numbers remain remarkably constant. To our knowledge, such an analysis of the frequency of TT-specific T cells has not been published before. Only in one donor there was a strong, transient increase in the number of TT-specific IFN-γ secreting cells during a flu-like illness. This increase may be due to unspecific *in vivo* stimulation and recruitment of TT-specific T cells for IFN-γ secretion, mediated by macrophage cytokines such as TNF-α. Recruitment of NK cells after initial activation of T helper cells is also possible. Crossreactivity with an infection-associated epitope and

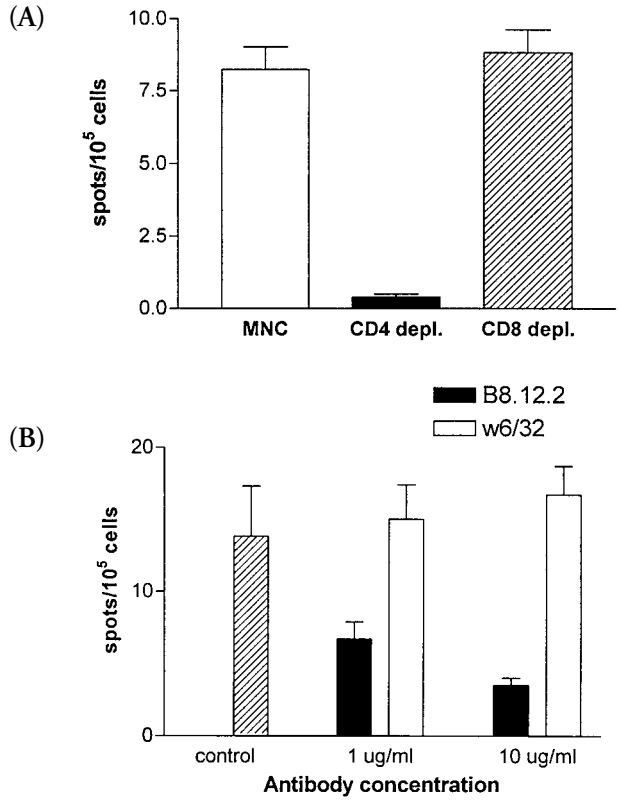

Figure 4. TT-reactive cells reside in the HLA-DR restricted $CD4^+$ T helper cell compartment. (**A**) The frequency of IFN-γ secreting cells in response to TT was analyzed either in PBMC or in CD4- or CD8-depleted cell fractions. Depletion of $CD4^+$ or $CD8^+$ cells was performed using magnetic beads as described in "Materials and Methods", with contaminating cells thereafter being < 2% of the population. Mean results of three independent experiments are shown. (**B**) PBMC were cultured for 20 hours in the presence of 3 μg/ml TT and 1 μg/ml or 10 μg/ml of either anti-HLA-DR mAB (B8.12.2), anti HLA-ABC mAB (W6/32) or without antibody (control).

consecutive amplification of TT-specific T cells may be another explanation (14, 15). The possibility that the obsereved IFN-γ secreting cells are not truely TT-specific rather are reacting to a contaminant (some bacterial product) present in the TT peparation used for the Elispot assay is less likely, since the purity of the TT preparation is high according to the information provided by the manufacturer. A nonspecific increase of IFN-γ production by T cells *in vitro* also does not seem likely since there was no significant increase in background IFN-γ secretion in the absence of antigen (data not shown).

All three donors receiving TT booster-immunizations showed a significant increase in the number of specific IFN-γ secreting T cells. After several weeks, a slow decline in TT-specific T cell frequencies could be observed, confirming the results of previous studies which analyzed shorter time periods and used different tools of analysis (16).

In previous reports, the Elispot assay has been established and been mainly used to assess the number of HLA class I antigen-restricted $CD8^+$ cytotoxic T cells (12, 17). However, the human TT-specific T cell response is mostly confined to HLA class II restricted $CD4^+$ T helper cells (10, 18), which is in accordance with our data shown above. *In vitro* data from KOZBOR et al. (3) suggest the additional existence of HLA-DR4 restricted TT-specific $CD8^+$ T cell clones. However, in our experiments there was no evidence for a significant contribution of TT-specific $CD8^+$ T cells to the total number of reactive cells since depletion of $CD8^+$ T cells led rather to an increase in the number of IFN-γ secreting T cells. There was a slight decrease in the number of IFN-γ secreting cells after the depletion of $CD16^+/CD56^+$ cells in addition to $CD8^+$ cells. This may either be due to positive feedback loops involving NK cells or by negative depletion of preactivated $CD56^+$ T cells. However, the strong residual activity within the $CD8^+/CD16^+/CD56^+$ depleted population and the almost complete abrogation of this activity after $CD4^+$ depletion corroborates the central role of $CD4^+$ T lymphocytes for IFN-γ production in our system.

Although the unexplained "booster" of TT-reactivity during a common cold of one donor leaves us with some questions about the specificity of the results in a situation of general immune activation, the stable quantities of IFN-γ secreting cells over time measured in the other donors and the specific increase after TT vaccination makes the results plausible from a biological point of view. In conclusion, our data show that the Elispot assay can be reliably used for the determination and monitoring of TT-specific T helper cells over time and may also be suitable for the quantification of T helper cells specific for other antigens. Furthermore, since most people are vaccinated against tetanus, quantitation of TT specific T helper cells by Elispot can be used as a positive control for the monitoring of specific T cell responses of patients in clinical protocols.

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References

1. WELLHÖRNER, H. H. 1981. Tetanus: important new concepts. In: Veronesi,R. (ed). Excerpta medica. Amsterdam, pp. 41.
2. DEMOTZ, S., P. M. MATRICARDI, C. IRLE, P. PANINA, A. LANZAVECCHIA, and G. CORRADIN. 1989. Processing of tetanus toxin by human antigen-presenting cells. Evidence for donor and epitope-specific processing pathways. J. Immunol. **143:** 3881–3886.
3. KOZBOR, D., G. TRINCHIERI, D. S. MONOS, M. ISOBE, G. RUSSO, J. A. HANEY, C. ZMIJEWSKI, and C. M. CROCE. 1989. Human TCR-gamma$^+$/delta$^+$, $CD8^+$ T lymphocytes recognize tetanus toxoid in an MHC-restricted fashion. J. Exp. Med. **169:** 1847–1851.
4. ADAMS, P. W., E. M. OPREMCAK, and C. G. OROSZ. 1991. Limiting dilution analysis of human, tetanus-reactive helper T lymphocytes. A rapid method for the enumeration of helper T lymphocytes with specificity for soluble antigens. J. Immunol. Methods **142:** 231–241.
5. GEHA, R. S., E. SCHNEEBERGER, F. S. ROSEN, and E. MERLER. 1973. Interaction of human thymus-derived and non-thymus-derived lymphocytes in vitro. Induction of proliferation and antibody synthesis in B lymphocytes by a soluble factor released from antigen-stimulated T lymphocytes. J. Exp. Med. **138:** 1230–1247.

6. KABILAN, L., G. ANDERSSON, F. LOLLI, H. P. EKRE, T. OLSSON, and M. TROYE-BLOMBERG. 1990. Detection of intracellular expression and secretion of interferon-gamma at the single-cell level after activation of human T cells with tetanus toxoid in vitro. Eur. J. Immunol. **20:** 1085–1089.
7. PARRONCHI, P., D. MACCHIA, M. P. PICCINNI, P. BISWAS, C. SIMONELLI, E. MAGGI, M. RICCI, A. A. ANSARI, and S. ROMAGNANI. 1991. Allergen- and bacterial antigen-specific T-cell clones established from atopic donors show a different profile of cytokine production. Proc. Natl. Acad. Sci. U. S. A **88:** 4538–4542.
8. DHODAPKAR, M. V., R. M. STEINMAN, M. SAPP, H. DESAI, C. FOSSELLA, J. KRASOVSKY, S. M. DONAHOE, P. R. DUNBAR, V. CERUNDOLO, D. F. NIXON, and N. BHARDWAJ. 1999. Rapid generation of broad T-cell immunity in humans after a single injection of mature dendritic cells. J. Clin. Invest. **104:** 173–180.
9. KELSO, A. 1989. Cytokines: structure, function and synthesis. Curr. Opin. Immunol. **2:** 215–225.
10. FERNANDEZ, V., J. ANDERSSON, U. ANDERSSON, and M. TROYE-BLOMBERG. 1994. Cytokine synthesis analyzed at the single-cell level before and after revaccination with tetanus toxoid. Eur. J. Immunol. **24:** 1808–1815.
11. SCHEIBENBOGEN, C., K. H. LEE, S. MAYER, S. STEVANOVIC, U. MOEBIUS, W. HERR, H. G. RAMMENSEE, and U. KEILHOLZ. 1997. A sensitive ELISPOT assay for detection of $CD8^+$ T lymphocytes specific for HLA class I-binding peptide epitopes derived from influenza proteins in the blood of healthy donors and melanoma patients. Clin. Cancer Res. **3:** 221–226.
12. LALVANI, A., R. BROOKES, S. HAMBLETON, W. J. BRITTON, A. V. HILL, and A. J. MCMICHAEL. 1997. Rapid effector function in $CD8^+$ memory T cells. J. Exp. Med. **186:** 859–865.
13. SCHMITTEL, A., U. KEILHOLZ, and C. SCHEIBENBOGEN. 1997. Evaluation of the interferon-gamma ELISPOT-assay for quantification of peptide specific T lymphocytes from peripheral blood. J. Immunol. Methods **210:** 167–174.
14. OLDSTONE, M. B. 1987. Molecular mimicry and autoimmune disease. Cell **50:** 819–820.
15. LOFTUS, D. J., C. CASTELLI, T. M. CLAY, P. SQUARCINA, F. M. MARINCOLA, M. I. NISHIMURA, G. PARMIANI, E. APPELLA, and L. RIVOLTINI. 1996. Identification of epitope mimics recognized by CTL reactive to the melanoma/melanocyte-derived peptide MART-1(27–35). J. Exp. Med. **184:** 647–657.
16. ELGHAZALI, G. E., S. PAULIE, G. ANDERSSON, Y. HANSSON, G. HOLMQUIST, J. B. SUN, T. OLSSON, H. P. EKRE, and M. TROYE-BLOMBERG. 1993. Number of interleukin-4- and interferon-gamma-secreting human T cells reactive with tetanus toxoid and the mycobacterial antigen PPD or phytohemagglutinin: distinct response profiles depending on the type of antigen used for activation. Eur. J. Immunol. **23:** 2740–2745.
17. BHARDWAJ, N., A. BENDER, N. GONZALEZ, L. K. BUI, M. C. GARRETT, and R. M. STEINMAN. 1994. Influenza virus-infected dendritic cells stimulate strong proliferative and cytolytic responses from human $CD8^+$ T cells. J. Clin. Invest **94:** 797–807.
18. DHODAPKAR, M. V., J. KRASOVSKY, R. M. STEINMAN, and N. BHARDWAJ. 2000. Mature dendritic cells boost functionally superior $CD8(^+)$ T-cell in humans without foreign helper epitopes. J. Clin. Invest. **105:** 9–14.

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