INFECTION AND IMMUNITY, Mar. 1992, p. 1229–1231
0019-9567/92/031229-03$02.00/0

Vol. 60, No. 3

Gamma Interferon and Interleukin 2, but Not Interleukin 4, Are Detectable in γ/δ T-Cell Cultures after Activation with Bacteria

GEORGE A. FOLLOWS, MARTIN E. MUNK, ALISON J. GATRILL, PETER CONRADT, AND STEFAN H. E. KAUFMANN*

Department of Immunology, University of Ulm, Albert-Einstein-Allee 11, D-7900 Ulm, Germany

Received 10 October 1991/Accepted 20 December 1991

***Mycobacterium tuberculosis*- or group A streptococcus-activated γ/δ T cells from normal healthy individuals were negatively sorted and restimulated in vitro for 48 h. Significant amounts of gamma interferon were detected after restimulation with *M. tuberculosis*, group A streptococci, or *Listeria monocytogenes*. In contrast, interleukin 4 was undetectable in the culture supernatants. Our findings provide indirect evidence for the involvement of γ/δ T cells in immunity against tubercle bacilli and probably other bacteria.**

Tuberculosis is a chronic infectious disease of worldwide clinical significance, primarily caused by *Mycobacterium tuberculosis* (12). T cells rather than antibodies play the dominant role in acquired resistance, and macrophage activation seems to be of major importance (9, 12). More recently, evidence has accumulated that not only T cells bearing an α/β T-cell receptor (TCR) but also γ/δ T cells may contribute to immunity to mycobacteria and many other bacteria, including the intracellular pathogen *Listeria monocytogenes* and the purulent group A streptococci (4–9, 11–13, 15–19). In vitro studies revealed that mycobacterium-activated γ/δ T cells express cytolytic activity and produce interleukin 2 (IL-2) as well as a still-undefined interleukin which synergizes with granulocyte macrophage-colony stimulating factor in macrophage aggregation (13, 16). The study described here shows that γ/δ T-cell cultures from healthy donors can produce gamma interferon (IFN-γ) and IL-2, but not detectable IL-4, after stimulation with *M. tuberculosis* and other bacteria. To demonstrate this, peripheral blood mononuclear cells (PBMC) from healthy donors with no history of tuberculosis and of unknown tuberculin status were isolated by Ficoll density gradient centrifugation (Biochrom, Berlin, Germany) at 5×10^6 cells per ml and were cultured for 8 to 11 days in RPMI 1640 medium and 10% heat-inactivated human A^+ Rh^+ serum with either killed *M. tuberculosis* (50 μg/ml) or group A streptococci (10^8/ml). From these cultures, γ/δ T cells were enriched by flow sorting as follows. Viable cells were purified over a Ficoll gradient, washed, passaged through nylon wool columns, and then labelled with anti-α/β TCR (BMA031, kindly provided by Behringwerke, Marburg, Germany) followed by goat anti-mouse $F(ab')_2$ immunoglobulin G-fluorescein isothiocyanate (Dianova) and Leu19-PE (CD56; Becton Dickinson, Heidelberg, Germany). Negatively stained cells were sorted and subsequently analyzed with fluorescein isothiocyanate-conjugated anti-TCR δ1 (T Cell Sciences, Cambridge, Mass.) by using an Epics V flow cytometer (Coulter). The mean purity of γ/δ T cells obtained after sorting was 83.5% ± 7.8%. Contamination with $BMA031^+$ $CD3^+$ TCR $\delta1^-$ cells was less than 1.5% ($x = 0.7 \pm 0.6$) in all samples which were assayed further.

Sorted γ/δ T cells (5×10^4/ml) were then cultured in 200 μl of medium with irradiated (4,000 rads) autologous accessory cells (2×10^5/ml) together with one of the following preparations: killed *M. tuberculosis* (25 μg/ml), killed group A streptococci (10^8/ml), killed *L. monocytogenes* (10^8/ml), or phytohemagglutinin (PHA) (5 μg/ml). After 48 h, supernatants were collected (100 μl per well), pooled, aliquoted, and frozen at −70°C until used. Triplicate cell cultures were pulsed for 16 h with 1 μCi of [^{3}H]thymidine per ml before cells were harvested and assayed for [^{3}H]thymidine incorporation. Anti-TCR δ1 (an ascites preparation kindly provided by M. Brenner; 1:200 dilution, used at 30 μl/ml) was added to some cultures as indicated in Tables 1 and 2 to suppress subsequent proliferation of γ/δ T cells.

The supernatant concentrations of the following cytokines were subsequently assayed by using the following enzyme-linked immunosorbent assay (ELISA) kits: IL-2 (R & D Systems) (minimum assay sensitivity, 150 pg/ml); IL-4 (R & D Systems) (minimum assay sensitivity, 5 to 10 pg/ml); IFN-γ (Holland Biotechnology) (minimum assay sensitivity, 15 pg/ml); tumor necrosis factor beta (TNF-β) (R & D Systems) (minimum assay sensitivity, 10 pg/ml). An IL-2-dependent mouse cell line (CTLL) was used for the IL-2 bioassay as described previously (16).

Consistent with previous data (16), purified γ/δ T cells which were originally expanded in bulk culture for 8 to 11 days with *M. tuberculosis* proliferated in vitro in response to the nominal antigen (mean counts per minute ± standard deviation = 2,251 ± 1,542), group A streptococci (8,728 ± 7,670), *L. monocytogenes* (1,803 ± 1,158), and PHA (10,877 ± 12,057). Control cultures showed no or marginal responses (95 ± 118). In contrast to responses of unsorted *M. tuberculosis*-stimulated PBMC, responses of γ/δ T cells to *M. tuberculosis* were markedly reduced in the presence of anti-TCR δ1 monoclonal antibody (MAb) (298 ± 202) (data not shown). These findings indicate negative effects of the anti-TCR δ1 MAb on γ/δ T-cell activation.

Following primary activation of PBMC in vitro with *M. tuberculosis* or group A streptococci, purified γ/δ T cells were restimulated for 2 days with *M. tuberculosis*, group A streptococci, *L. monocytogenes*, or PHA, and the culture supernatants were collected for cytokine production analyses. In none of the eight donors tested was IL-4 detectable after the various stimulations (data not shown). We interpret this finding to indicate that mycobacterium-activated γ/δ T cells fail to secrete demonstrable amounts of IL-4, although we cannot formally exclude the possibility that appreciable concentrations of IL-4 were produced and subsequently

* Corresponding author.

taken up by the cells in culture. In contrast, most γ/δ T-cell cultures produced significant amounts of IFN-γ when restimulated with *M. tuberculosis*, group A streptococci, or *L. monocytogenes* (Table 1). PHA induced accentuated IFN-γ production in most donors. Preincubation with anti-TCR δ1 MAb was found to inhibit IFN-γ secretion in all samples assayed, confirming the involvement of γ/δ T cells in IFN-γ production. Cultures from four of the eight donors tested produced low but detectable amounts of TNF-β following bacterial stimulation, whereas PHA activation caused appreciably higher levels of TNF-β activity (Table 2). Interestingly, no correlation between IFN-γ and TNF-β production was observed with these donors. Because relatively low concentrations of TNF-β were detectable and since we failed to demonstrate significant reduction of TNF-β production by the addition of the TCR δ1 MAb, formal proof for γ/δ T-cell involvement cannot be given.

In none of these cultures was IL-2 activity detectable by ELISA. Employing the CTLL bioassay as previously described (16), we found various amounts of IL-2 after stimulation with *M. tuberculosis* (mean counts per minute ± standard deviation, 8,504 ± 15,333), group A streptococci (7,171 ± 13,626), and PHA (15,956 ± 14,840). Accessory cells alone did not induce IL-2 (79 ± 70). Yet the largest amount of IL-2 activity detected was in the range of 0.5 U/ml, a level of activity which is below ELISA sensitivity range (150 to 200 pg/ml ≈ 0.45 to 0.6 U/ml).

Immunization of mice with killed mycobacteria as well as infection with viable *Mycobacterium bovis* BCG and *L. monocytogenes* causes attraction of γ/δ T cells to, and/or expansion at, the site of bacterial implantation (6, 7, 18). Furthermore, in certain lesions in patients with leprosy and tuberculosis and at the site of lepromin reactions, γ/δ T cells have been identified (4, 13). Taken together, these findings suggest a role for γ/δ T cells in immunity to mycobacteria and perhaps other bacteria. However, the biological function(s) of such γ/δ T cells remains elusive. The experiments presented here show that in vitro activation of purified γ/δ T cells with *M. tuberculosis* or group A streptococci results in production of IFN-γ, which is thought to be of major importance for antimycobacterial immunity.

Both in the human and in the murine systems, mycobacterium-reactive α/β T cells express the T_{H1} phenotype, and overwhelming evidence suggests that these T_{H1} cells and their major secretion product, IFN-γ, play the central part in protection (1–3, 10, 14, 20, 21). According to our experiments, mycobacterium-activated γ/δ T cells resemble CD4 T cells of the T_{H1} type. We assume that both T-cell types contribute to antibacterial immunity through similar functional activities, i.e., IFN-γ secretion and target cell lysis. The rapid expansion of human γ/δ T cells after in vitro activation by mycobacterial components as well as the early appearance of γ/δ T cells in experimental listeriosis and tuberculosis (6, 18) indicates that γ/δ T cells precede α/β T cells at the site of bacterial growth at which they could provide a first line of antibacterial defense.

This work received financial support to S.H.E.K. from the EEC-India Science and Technology Cooperation Program, the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases, the German Leprosy Relief Association, the A. Krupp Award for young professors, and the Deutsche Forschungsgemeinschaft (SFB 322). G.A.F. was supported by a scholarship from the British Chamber of Commerce in Germany and a grant from Worcester College, Oxford, England, and A.J.G. was supported by a Wellcome Research fellowship.

TABLE 1. IFN-γ production by γ/δ T-cell cultures

Secondary stimulant	Amt/ml	IFN-γ production by γ/δ T-cell cultures from different donors[a]									
		1M	2M	3M	3S	4M	4S	5M	6M	7M	8M
M. tuberculosis	25 μg	<T	<T	17 ± 1	<T	260 ± 71	185 ± 21	95 ± 0	79 ± 1	39 ± 7	<T
M. tuberculosis plus TCR δ1 MAb[b]	25 μg	<T	<T	<T	<T	<T	<T	ND	<T	<T	<T
Group A streptococci	10^8	<T	<T	36 ± 5	38 ± 7	205 ± 64	250 ± 0	270 ± 14	840 ± 71	220 ± 0	350 ± 14
L. monocytogenes	10^8	<T	ND	ND	<T	120 ± 14	ND	ND	ND	85 ± 20	90 ± 28
PHA	5 μg	525 ± 0	<T	ND	25 ± 11	460 ± 70	525 ± 106	185 ± 35	2,400 ± 0	435 ± 160	1,300 ± 130
Accessory cells plus *M. tuberculosis*	25 μg	<T	<T	<T	<T	<T	<T	<T	<T	<T	<T

[a] Values are mean concentrations of IFN-γ ± standard deviations and are given as picograms per milliliter. PBMC were first stimulated with *M. tuberculosis* (M) (50 μg/ml) or group A streptococci (10^6/ml) (S); numbers designate different donors. <T, below threshold value (15 pg/ml); ND, not done.

[b] Secondary stimulation of sorted γ/δ T cells (5×10^4/ml) in the presence of TCR δ1 MAb (30 μl/ml diluted at 1:200).

TABLE 2. TNF-β production by γ/δ T-cell cultures

Secondary stimulant	Amt/ml	TNF-β production by γ/δ T-cell cultures from different donors[a]									
		1M	2M	3M	3S	4M	4S	5M	6M	7M	8M
M. tuberculosis	25 μg	28 ± 3	27 ± 13	<T	<T	<T	<T	<T	<T	<T	<T
M. tuberculosis plus TCR δ1 MAb[b]	25 μg	ND	37 ± 1	<T	<T	<T	<T	ND	<T	<T	<T
Group A streptococci	10^8	<T	30 ± 11	<T	<T	<T	<T	<T	14 ± 1	<T	18 ± 2
L. monocytogenes	10^8	19 ± 0	ND	ND	<T	<T	ND	ND	ND	ND	ND
PHA	5 μg	138 ± 4	<T	<T	<T	<T	<T	<T	195 ± 7	90 ± 0	140 ± 4
Accessory cells plus *M. tuberculosis*	25 μg	<T	<T	<T	<T	<T	<T	<T	<T	<T	<T

[a] Values are mean concentrations of TNF-β ± standard deviations and are given as picograms per milliliter. PBMC were first stimulated with *M. tuberculosis* (M) (50 μg/ml) or group A streptococci (S) (10^8/ml); numbers designate different donors. <T, below threshold value (10 pg/ml); ND, not done.
[b] Secondary stimulation of sorted γ/δ T cells (5×10^4/ml) in the presence of TCR δ1 MAb (30 μl/ml diluted at 1:200).

We thank R. Mahmoudi for secretarial help. We also thank M. Brenner and R. Kurrle for valuable reagents.

REFERENCES

1. **Barnes, P. F., S.-J. Fong, P. J. Brennan, P. E. Twomey, A. Mazumder, and R. L. Modlin.** 1990. Local production of tumor necrosis factor and IFN-γ in tuberculous pleuritis. J. Immunol. **145:**149–154.
2. **Cooper, C. L., C. Mueller, T.-A. Sinchaisri, C. Pirmez, J. Chan, G. Kaplan, S. M. M. Young, I. L. Weissman, B. R. Bloom, T. H. Rea, and R. L. Modlin.** 1989. Analysis of naturally occurring delayed type hypersensitivity reactions in leprosy by in situ hybridization. J. Exp. Med. **169:**1565–1581.
3. **Emmrich, F., J. Thole, J. van Embden, and S. H. E. Kaufmann.** 1986. A recombinant 64 kiloDalton protein of *Mycobacterium bovis* BCG specifically stimulates human T4 clones reactive to mycobacterial antigens. J. Exp. Med. **163:**1024–1029.
4. **Falini, B., L. Flenghi, S. Pileri, P. Pelicci, M. Fagiolo, M. F. Martelli, L. Moretta, and E. Ciccone.** 1989. Distribution of T cells bearing different forms of the T cell receptor γ/δ in normal and pathological human tissues. J. Immunol. **143:**2480–2488.
5. **Haregewoin, A., G. Soman, C. R. Hom, and R. W. Finberg.** 1990. Human γ/δ^+ T cells respond to mycobacterial heat-shock protein. Nature (London) **340:**309–312.
6. **Inoue, T., Y. Yosunobu, G. Matsuzaki, and K. Nomoto.** 1991. Early appearing γ/δ-bearing T cells during infection with Calmette Guérin bacillus. J. Immunol. **146:**2754–2762.
7. **Janis, E. M., S. H. E. Kaufmann, R. H. Schwartz, and D. M. Pardoll.** 1989. Activation of γ/δ^+ T cells in the primary immune response to *Mycobacterium tuberculosis*. Science **244:**713–716.
8. **Kabelitz, D., A. Bender, S. Schondelmaier, B. Schoel, and S. H. E. Kaufmann.** 1990. A large fraction of human peripheral blood γ/δ^+ T cells is activated by *Mycobacterium tuberculosis* but not by its 65 kD heat shock protein. J. Exp. Med. **171:**667–679.
9. **Kaufmann, S. H. E.** 1988. $CD8^+$ T lymphocytes in intracellular microbial infections. Immunol. Today **9:**168–174.
10. **Kaufmann, S. H. E., and I. Flesch.** 1986. Function and antigen recognition pattern of $L3T4^+$ T-cell clones from *Mycobacterium tuberculosis*-immune mice. Infect. Immun. **54:**291–296.
11. **Kaufmann, S. H. E., and D. Kabelitz.** 1991. Gamma/delta T lymphocytes and heat shock proteins. Curr. Top. Microbiol. Immunol. **167:**191–207.
12. **Kaufmann, S. H. E., and D. B. Young.** Vaccination against tuberculosis and leprosy. Immunobiology, in press.
13. **Modlin, R. L., C. Pirmez, F. M. Hofmann, V. Torigian, K. Uyemura, T. H. Rea, B. R. Bloom, and M. B. Brenner.** 1989. Lymphocytes bearing antigen-specific γ/δ T cell receptors accumulate in human infectious disease lesions. Nature (London) **339:**544–548.
14. **Mosmann, T. R., and R. L. Coffman.** 1989. T_{H1} and T_{H2} cells: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. **7:**145–174.
15. **Müller, I., S. P. Cobbold, H. Waldmann, and S. H. E. Kaufmann.** 1987. Impaired resistance to *Mycobacterium tuberculosis* infection after selective in vivo depletion of $L3T4^+$ and $Lyt\text{-}2^+$ T cells. Infect. Immun. **55:**2037–2041.
16. **Munk, M. E., A. Gatrill, and S. H. E. Kaufmann.** 1990. Antigen specific target cell lysis and interleukin-2 secretion by *Mycobacterium tuberculosis* activated γ/δ T cells. J. Immunol. **145:**2434–2439.
17. **O'Brien, R. L., M. P Happ, A. Dallas, E. Palmer, R. Kubo, and W. Born.** 1989. Stimulation of a major subset of lymphocytes expressing T cell receptor γ/δ by an antigen derived from *Mycobacterium tuberculosis*. Cell **57:**667–674.
18. **Ohga, S., Y. Yoshikai, Y. Takeda, K. Hiromatsu, and K. Nomoto.** 1990. Sequential appearance of γ/δ- and α/β-bearing T cells in the peritoneal cavity during an i.p. infection with *Listeria monocytogenes*. Eur. J. Immunol. **20:**533–538.
19. **Pfeffer, K., B. Schoel, H. Gulle, S. H. E. Kaufmann, and H. Wagner.** 1990. Primary responses of human T cells to mycobacteria: a frequent set of γ/δ T cells are stimulated by protease-resistant ligands. Eur. J. Immunol. **20:**1175–1179.
20. **Romagnani, S.** 1991. Human T_H1 and T_H2 subsets: doubt no more. Immunol. Today **12:**256–257.
21. **Wierenga, E. A., M. Snoel, H. M. Jansen, J. D. Bos, R. A. W. van Lier, and M. L. Kapsenberg.** 1991. Human atopen-specific types 1 and 2 T helper cell clones. J. Immunol. **147:**2942–2949.