CORRESPONDENCE

This department is for the publication of informal communications that are of interest because they are informative and stimulating, and for the discussion of controversial matters. The mandate of this JOURNAL is to disseminate information relating to leprosy in particular and also other mycobacterial diseases. Dissident comment or interpretation on published research is of course valid, but personality attacks on individuals would seem unnecessary. Political comments, valid or not, also are unwelcome. They might result in interference with the distribution of the JOURNAL and thus interfere with its prime purpose.

Unresponsiveness to *Mycobacterium leprae* in Lepromatous Leprosy *in Vitro*: Reversible or Not?

TO THE EDITOR:

The absence of detectable cellular immune reactivity either *in vivo* by skin testing ([11]) or *in vitro* by lymphocyte transformation tests ([1, 4, 12]) in lepromatous leprosy is well documented. However, much less is known about the mechanism(s) which determine(s) immunological host unresponsiveness. Many reports have been published on the *in vitro* suppression of proliferative responses by *Mycobacterium leprae* (as reviewed in [3, 10]). Evidence for active and specific immunosuppression in lepromatous leprosy patients by suppressor-cells and -factors has been presented. Haregewoin, *et al.* ([6]) recently reported a defect in the *in vitro* production of interleukin 2 (IL2) in *M. leprae*-stimulated cultures of peripheral blood mononuclear cells (PBMNC) of lepromatous leprosy patients resulting in nonresponsiveness. This defect could be overcome by the addition of IL2 which restored the *M. leprae*-specific proliferative response in at least 13 of 17 patients tested (4 BL, 13 LL).

The way in which suppressor cells exert their influence is the subject of intense investigation. T-suppressor cells and -products have been described to inhibit IL2 production ([5, 8, 9]). Obviously, it would have important practical implications with regard to therapy if immunosuppression could be circumvented. Therefore, we have repeated this study and we report here our findings in 12 patients. In only two patients have we been able to reverse the unresponsiveness toward *M. leprae*; whereas the other ten showed no significant increase in response.

Twelve patients, 6 BL (Nos. 1–6) and 6 LL (Nos. 7–12), were selected in collaboration with Dr. D. L. Leiker (Royal Tropical Institute, Amsterdam); 9 patients originated from the Caribbean area, 3 from eastern Asia. The mean duration of treatment was 13.8 ± 9.4 years (ranging from 1.0–28.0 years). All patients were receiving combination therapy; 9 out of 12 had received triple therapy for one year (i.e., dapsone + clofazimine + rifampin).

PBMNC were isolated from fresh heparinized blood via a Ficoll-Isopaque gradient, washed three times, and resuspended in a medium consisting of Iscove's modified Dulbecco's medium and 20% heat-inactivated, pooled human AB serum in a concentration of 1×10^6 or 1.5×10^6 or 2×10^6 cells/ml. Suspensions were divided into equal aliquots, to only one of which IL2 was added in a concentration of 2% (Lymphocult T, Biotest). Different *M. leprae* preparations were tested in optimal concentrations as assessed by maximal proliferative responses of tuberculoid patients (BT and TT) (data not shown). Dharmendra antigen was prepared by Dr. M. Abe, Tokyo. PL and BZ were both prepared by Dr. P. Draper, London. PL was purified according to IMMLEP Protocol 1/79. BZ was purified in the same way but with the addition of 1 mM benzamidine hydrochloride and 1 mM $MgSO_4$ to the homogenizing buffer. Lep-

TABLE 1. *The proliferation of peripheral blood mononuclear cells from lepromatous*

Antigen	None		Dharmendra		PL
IL2	−	+	−	+	−
Patient no.					
1	445 ± 160[a]	2030 ± 487	1025 ± 328 (2.3)[b]	6943 ± 1875 (3.4)	851 ± 153 (1.9)
2	535 ± 107	996 ± 249	528 ± 26 (1.0)	3336 ± 1068 (3.4)	350 ± 42 (0.7)
3	186 ± 71	596 ± 89	361 ± 69 (1.9)	543 ± 244 (0.9)	530 ± 291 (2.9)
4	931 ± 140	2906 ± 262	1228 ± 221 (1.4)	3705 ± 1186 (1.3)	755 ± 76 (0.8)
5	2641 ± 1954	3266 ± 2152	1996 ± 279 (0.8)	2981 ± 477 (0.9)	2096 ± 587 (0.8)
6	413 ± 124	1418 ± 482	240 ± 53 (0.6)	2375 ± 143 (1.7)	566 ± 68 (1.4)
7	910 ± 55	1725 ± 104	2186 ± 721 (2.4)	3678 ± 368 (2.1)	2091 ± 293 (2.3)
8	945 ± 454	2048 ± 1352	96 ± 62 (0.1)	1773 ± 372 (0.9)	850 ± 502 (0.9)
9	313 ± 185	375 ± 98	630 ± 95 (2.0)	1478 ± 414 (3.9)	1128 ± 237 (3.6)
10	573 ± 401	1297 ± 182	1177 ± 235 (2.0)	3190 ± 670 (2.5)	NT[c]
11	685 ± 123	1045 ± 146	755 ± 166 (1.1)	802 ± 209 (0.8)	NT
12	1642 ± 384	1585 ± 290	3565 ± 778 (2.2)	3382 ± 93 (2.1)	2538 ± 716 (1.0)

[a] Results expressed in mean cpm ± S.D.

[b] Stimulation Index = $\frac{\text{mean cpm with antigen}}{\text{mean cpm without antigen}}$.

[c] NT = not tested.

romin A was prepared according to IMMLEP Protocol 1/79. The suspensions were prepared in Iscove's medium in a concentration of 10^6–10^7 acid-fast bacilli/ml. In some but not all patients the supernatant of homogenized armadillo tissue (containing no bacilli, but large amounts of leprosy-specific glycolipids[2, 7]) was also tested in a wide concentration range (10^{-4}–2×10^{-1}). As controls, we also tested PPD (Statens Serum Inst., Copenhagen) (10 μg/ml); PHA (Wellcome) (4 μg/ml); and medium without antigen; 1×10^5, 1.5×10^5, or 2×10^5 cells (0.1 ml) and 0.1 ml of antigen suspension/solution were cultured in triplicate in 96-well, flat- or round-bottom plates for 5 days at 37°C in a humidified 5% CO_2 environment. One μCi tritium-labelled thymidine was then added to each culture. After an additional 16 hr, the cultures were harvested. Thymidine incorporation as a measure of cell proliferation was measured by liquid scintillation counting. Results are shown in Table 1 expressed in counts per minute (cpm) ± the standard deviation (S.D.) and stimulation indices (S.I.).

In all patients the positive control proliferations (PHA) were strongly positive with or without the addition of IL2 (data not shown). In only one patient (BL) (No. 1) was the proliferative response against *M. leprae* clearly increased after the addition of IL2 (pooled data from Dharmendra, PL and BZ: cpm increased from 983 to 13,652, S.I. from 2.0 to 6.7). This was specific for *M. leprae* because it was not observed in the case of PPD. In one other BL patient (No. 2) a similar but less clearcut phenomenon was seen (pooled: cpm increased from 663 to 3795, S.I. from 1.3 to 3.8). In this case also the response to PPD was increased by IL2. Data for patients Nos. 1 and 2 for Dharmendra, PL and BZ antigens are pooled in Table 2. In the 10 other patients (4 BL, 6 LL) (Nos. 3–12) no significant differences could be found in responsiveness after the addition of IL2. Proliferation increased both in the presence and in the absence of *M. leprae,* resulting in equal stimulation indices. Data pooled for Dharmendra, PL and BZ antigens in patients Nos. 3–12 show a twofold increase in cpm in the presence of *M. leprae* antigen after the addition of IL2, but this is not specific because this is also seen in cultures in the absence of *M. leprae* antigen. Therefore there is no change in the stimulation index (Table 3). Data concerning Lepromin A and supernatant are not shown; in none of the patients tested were differences in proliferation seen to these *M. leprae* preparations in the absence or presence of IL2.

These results indicate that it is possible to reverse *in vitro* the specific unresponsiveness to *M. leprae* in some lepromatous patients, but not in the majority. This is in

(BL and LL) patients in response to antigens in the presence and absence of interleukin 2.

PL	BZ		PPD	
+	−	+	−	+
14,536 ± 5233 (7.2)	893 ± 223 (2.0)	19,478 ± 6038 (9.6)	1041 ± 458 (2.3)	2093 ± 586 (1.0)
3863 ± 927 (3.9)	1110 ± 111 (2.1)	4185 ± 711 (4.2)	1691 ± 676 (3.2)	5720 ± 3089 (5.7)
520 ± 104 (0.9)	421 ± 88 (2.3)	350 ± 21 (0.6)	421 ± 13 (2.3)	760 ± 342 (1.3)
4156 ± 42 (1.4)	691 ± 41 (0.7)	3330 ± 100 (1.2)	1220 ± 500 (1.3)	4075 ± 530 (1.4)
4311 ± 992 (1.3)	2183 ± 240 (0.8)	4983 ± 897 (1.5)	3876 ± 1395 (1.5)	4101 ± 574 (1.3)
2653 ± 80 (1.9)	715 ± 300 (1.7)	2855 ± 286 (2.0)	600 ± 54 (1.5)	3060 ± 92 (2.2)
2951 ± 620 (1.7)	2455 ± 270 (2.7)	4243 ± 1443 (2.5)	2768 ± 609 (3.0)	2871 ± 144 (1.7)
2035 ± 468 (1.0)	1131 ± 339 (1.2)	2191 ± 372 (1.1)	1091 ± 371 (1.2)	2065 ± 124 (1.0)
1263 ± 278 (3.4)	1687 ± 186 (5.4)	1118 ± 358 (3.0)	2530 ± 961 (8.0)	1612 ± 258 (4.3)
NT	NT	NT	21,470 ± 2576 (37.5)	24,283 ± 4128 (18.7)
NT	NT	NT	1707 ± 785 (2.5)	4507 ± 2839 (4.3)
1615 ± 204 (1.0)	NT	NT	6760 ± 1795 (4.1)	15,338 ± 755 (9.7)

contrast with the recent findings of Haregewoin, *et al.* ([6]) who found a specific reversal into responsiveness in at least 13 out of 17 patients. The reason for this discrepancy is not clear to us. Slightly different technical procedures do not seem to contribute to this because we were able to demonstrate responsiveness in two patients. The same applies to the longer duration of treatment in our patients. In addition, Haregewoin, *et al.* found no differences correlated to the period of treatment. We conclude that there exists an apparent heterogeneity in the response to the addition of IL2 *in vitro* among leprosy patients. This could have important implications for the therapy of lepromatous leprosy patients with regard to the possible *in vivo* reversal of unresponsiveness against *M. leprae.*

There are several possible explanations to account for this heterogeneity: a) Suppression could be too strong to be circumvented by relatively small amounts of IL2 in the nonresponsive patients. This seems rather unlikely because we were not able to observe the effect even in the presence of 5% IL2. b) Another possibility is a difference in the mycobacterial environment patients have been exposed to, leading to a differential response to *M. leprae* in case suppression is abolished. This might be reflected in the much lower responses of our patients tested against PPD, in comparison to the Ethiopian leprosy patients. c) Heterogeneity in genetic background of different populations might result in differences in immunoregulatory mechanisms, quantitatively or qualitatively. Different suppressor cells and factors can be involved, operating in several ways, only one of which in that case would be regulation of IL2 production. Interestingly, two studies recently have suggested the existence of HLA-linked genes predisposing to lepromatous leprosy. This was based on the HLA haplotype segregation analysis in multicase lepromatous leprosy families in both Venezuela ([13]) and China (Xu Keju, *et al.*, submitted for publication).

TABLE 2. *Mean cpm ± S.D. and mean stimulation indices (S.I.) ± S.D. of patients Nos. 1 and 2. Data for* M. leprae *antigens Dharmendra, PL and BZ are pooled.*

		Interleukin 2	
		−	+
M. leprae	+	793 ± 295	8724 ± 6721
	−	490 ± 64	1513 ± 731
S.I.		1.67 ± 0.65	5.28 ± 2.55

TABLE 3. *Mean cpm ± S.D. and mean stimulation indices (S.I.) ± S.D. of patients Nos. 3–12. Data for* M. leprae *antigens Dharmendra, PL and BZ are pooled.*

		Interleukin 2	
		−	+
M. leprae	+	1283 ± 886	2499 ± 1322
	−	924 ± 732	1626 ± 921
S.I.		1.74 ± 1.13	1.66 ± 0.86

It would be of importance of perform similar studies in other populations to further elucidate the mechanism(s) by which suppression is exerted to evaluate the potential use of these findings with regard to therapy.

—Tom H. M. Ottenhoff, M.D.
—Dienne G. Elferink
—René R. P. de Vries, Ph.D.

Department of Immunohematology and Bloodbank
Academisch Ziekenhuis Leiden
Rijnsburgerweg 10
2333 AA Leiden
The Netherlands

REFERENCES

1. Bjune, G., Barnetson, R. St. C., Ridley, D. S. and Kronvall, G. Lymphocyte transformation test in leprosy: Correlation of the response with inflammation of lesions. Clin. Exp. Immunol. **25** (1976) 85–94.
2. Draper, P. Studies on *Mycobacterium leprae* from infected armadillos. In: *Leprosy.* Proc. XI International Leprosy Congress. Latapi, F., *et al.*, eds. Amsterdam: Excerpta Medica, 1980, p. 97.
3. Godal, T. Immunological aspects of leprosy—present status. Prog. Allergy **25** (1978) 211–242.
4. Godal, T., Myklestad, B., Samuel, D. R. and Myrvang, B. Characterization of the cellular immune defect in lepromatous leprosy: A specific lack of circulating *Mycobacterium leprae*-reactive lymphocytes. Clin. Exp. Immunol. **9** (1971) 821–831.
5. Gullberg, M. and Larrson, E. L. Studies on induction and effector functions of concanavalin A-induced suppressor cells that limit TCGF production. J. Immunol. **128** (1982) 746–750.
6. Haregewoin, A., Godal, T., Mustafa, A. S., Belehu, A., and Yemaneberhan, T. T cell conditioned media reverse T cell unresponsiveness in lepromatous leprosy. Nature **303** (1983) 342–344.
7. Hunter, S. W. and Brennan, P. J. A novel phenolic glycolipid from *Mycobacterium leprae* possibly involved in immunogenicity and pathogenicity. J. Bacteriol. **147** (1981) 728–735.
8. Kramer, M. and Koszinowski, U. T cell-specific suppressor factor(s) with regulatory influence on interleukin 2 production and function. J. Immunol. **128** (1982) 784–790.
9. Malkovsky, M., Asherson, G. L., Stockinger, B. and Watkins, M. C. Nonspecific inhibitor released by T acceptor cells reduces the production of interleukin-2. Nature **300** (1982) 652–655.
10. Nath, I. Immunology of human leprosy—current status. Lepr. Rev. Special Issue (1983) 31S–45S.
11. Rees, R. J. W. The significance of the lepromin reaction in man. Prog. Allergy **8** (1964) 224–258.
12. Turk, J. L. and Bryceson, A. D. M. Immunological phenomena in leprosy and related diseases. Adv. Immunol. **13** (1971) 209–266.
13. Van Eden, W. *HLA and leprosy. A model for the study of genetic control of immune response in a man,* thesis, Leiden, 1983.

Response to Letter of Dr. Ottenhoff, *et al.*

To the Editor:

We are grateful for your invitation to comment on the Letter to the Editor from Ottenhoff, *et al.* As discussed by Ottenhoff, *et al.*, the discrepancies between our published data (Haregewoin, *et al.*, Nature **303** [1983] 342–344) and theirs may be due to either methodological differences or differences in the patient groups.

With the kind assistance of Dr. Leiker, we have had the opportunity of studying several patients from Holland. Our results are shown in Table 1. As can be seen from Table 1, we have also found a poor response of such patients to T-cell conditioned medium (TCM) (Lymphocult T, Biotest). Only 2 out of 8 patients gave a significant response (>5000 Δ cpm). However, both of the responders also responded to *M. leprae* alone. Thus, our data with TCM are quite analogous to those of Ottenhoff, *et al.* The only notable difference between their data and ours is that, while in their assay the overall response to PPD was quite low, we had good responses to BCG in the three patients tested. This difference may indicate that significant methodological differences exist between our two laboratories, but their relevance to the interleukin 2 (IL2) effect in lepromatous leprosy remains unclear. In contrast to TCM, recombinant IL2 appears to have significant effects in 3 out of 4 tested patients. We hope that Ottenhoff, *et al.* will have the opportunity also to study recombinant IL2. We agree with Ottenhoff, *et al.* that the most likely explanation for the dis-