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Research Note

HUMAN MONOKINE PARTICIPATION IN MURINE THYMOCYTE PROLIFERATION

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In addition to interleukin 1 (IL-1), the first cytokine described as having co-mitogenic activity for murine thymocytes (I. Gery et al., 1972, *J. Exp. Med., 136:* 128-142), there are several cytokines such as interleukin 2 (IL-2), interleukin 6 (IL-6), murine tumor necrosis factor alpha (murine TNF) and interleukin 7 that are now known to induce the proliferation of bulk suspensions of murine thymocytes, either in the presence or in the absence of lectins (J. Shaw et al., 1978, *J. Immunol, 120:* 1967-1973; L. Lotz et al., 1988, *J. Exp. Med., 167:* 1253-1258; G. E. Ranges et al., 1988, *J. Exp. Med., 167:* 1472-1478; P. J. Conlon et al., 1989, *Blood, 74:* 1368-1373).

Using the murine thymocyte co-stimulatory assay for the measurement of cytokine secretion induced *in vitro* by mononuclear adherent cells (monocyte/dendritic cells) derived from peripheral blood of patients with rheumatoid arthritis (RA), we found that RA-derived cells do not need stimulation to secrete a thymocyte comitogenic activity (K. C. Sabino et al., 1989, 7th Intern. Congress Immunol., Berlin, abstract 83-61). The comitogenic activity found in such non-stimulated as well as in LPS-stimulated incubation media (conditioned media, CM) has been ascribed to cytokines known to be produced by these cells, such as IL-1, IL-6 and TNF, alone or combined, or it may involve other cytokines not yet described.

It has been shown that murine thymocyte proliferation is not co-stimulated by human TNF (G. E. Ranges et al., *loc. cit.*). However, in that study, the possibility could not be ruled out that human TNF can contribute to CM activity when combined with other cytokines. This hypothesis is supported by the fact that human and murine TNF compete for binding to murine thymocytes (G. E. Ranges et al., *loc. cit.*) and that the TNF molecule bears two different binding sites, probably for two different kinds of receptors on target cells (O. Stutman, personal communication).

In this report, we use thymocytes prepared from Balb/c mice to compare the proliferative response to supernatant (CM) from LPS-induced monocytes with the response to pure recombinant human cytokines, alone or combined. Supernatant from LPS-induced monocytes was prepared as described before (S. R. Dalmau & C. S. Freitas, 1990a, *Mem. Inst. Oswaldo Cruz, 85:* 153-161).

In the absence of lectin, only IL-1, from 0.1 to 10 units/well, was able to substantially stimulate thymocyte proliferation (not shown). In the presence of suboptimal doses of phytohemagglutinin (PHA) (for methods see: S. R. Dalmau & C. S. Freitas, 1990, *Mem. Inst. Oswaldo Cruz, 85:* 123-125), a significant costimulatory activity ($P < 0.025$) was observed for both IL-1, from 0.01 to 0.1 u/well, and IL-6 in the highest amount used (4500 u/well) (Fig. 1.A). Up to the highest dose tested (100 u/well, i.e., 100 ng/well) TNF alone failed to co-stimulate thymocyte proliferation (Fig. 1.A).

When combined two by two in the presence of PHA, only the highest amount of IL-6 exerted a cooperative effect with IL-1 (different from IL-1 alone with $P < 0.01$). Fig. 1.B-D shows that the addition of TNF had very little effect on the proliferation curves observed with IL-1 plus IL-6, although some points (marked with asterisks, box B) differ statistically from the control without TNF (box E)

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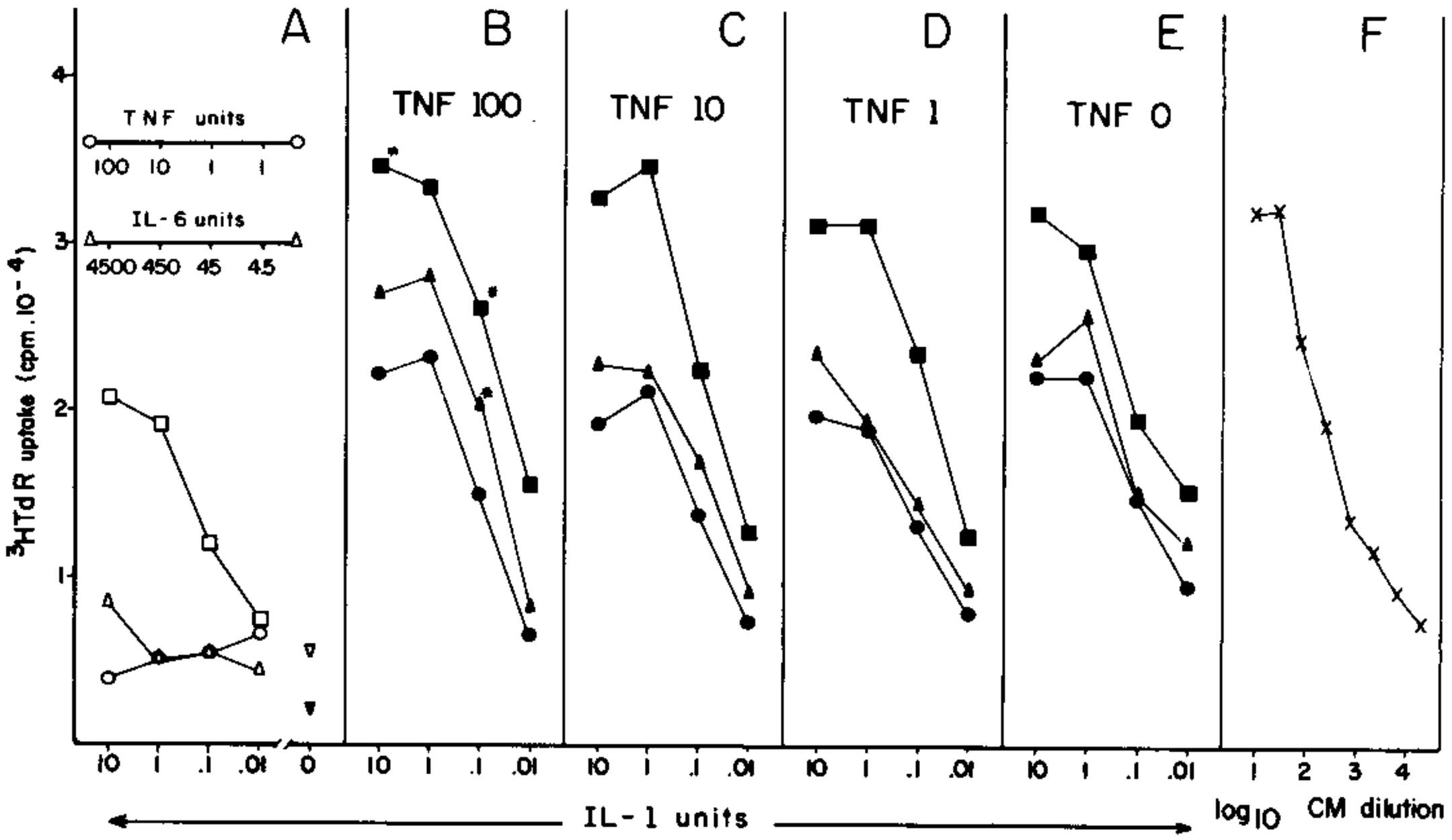

Proliferative response of murine thymocytes to three human cytokines, alone and in combination. Recombinant cytokines were added in log dilutions (shown in units/well) to bulk murine Balb/c thymocytes ($1.5x10^6$/well) in the presence of PHA, and proliferation was assessed by measuring ^{3}HTdR uptake for the last 24 h of a 72 h culture period. A. Individual cytokines: IL-1 (□), IL-6 (△) and TNF (○); and thymocytes in the absence of cytokines, with (△) or without (▲) PHA*. B-E. IL-6 at 45 (●), 450 (▲) or 4500 (■) u/well in the presence of IL-1 units shown on the abscissa, and TNF, 0-100 u/well as shown at the top of each box; asterisks represent significant increases ($P < 0.05$) compared to results for the same IL-1 and IL-6 amounts in E (no TNF). F. LPS-stimulated CM in a serial 3-fold dilution, starting from 1:9, in a log plot. The IL-6 content of this CM ranged from 2200 u/well in the first dilution (1:9) to aprox. 1 u/well in the last dilution (1:19683), as assessed by the B9 proliferation assay.
*Abscissae for IL-6 and TNF curves are shown in the upper part of panel A.

($P < 0.05$). Fig. 1.F shows that the maximum proliferation and slope of the dose-response curve obtained with LPS-induced CM closely resembled that observed for the cooperative effect of IL-1 plus IL-6 (4500 u/well) (Fig. 1.E).

In agreement with previous reports (P. J. Conlon et al., 1982, *J. Immunol., 128:* 797-801; M. Lotz et al., *loc. cit.*), an anti-IL-2-αchain-receptor antibody, PC61, inhibited by more than 90% the thymocyte proliferation in response to either IL-1, IL-6 or IL-1 plus IL-6, and the same inhibition occurred with the response to CM (not shown). These data suggest an IL-2 involvement in virtually all the thymocyte proliferative effect exerted by these cytokines or by crude supernatant.

Here, human TNF was shown to be ineffective as a co-mitogen for murine thymocytes, either alone or in combination with IL-1, IL-6 or both of these cytokines. Lack of stimulation was evident even with TNF concentrations as high as 500 ng/ml, far above the TNF concentrations usually found in LPS-stimulated monocyte supernatants (less than 5 ng/ml, as inferred from the biological assay using the WEHI-164 cell line and pure recombinant TNF as a standard).

The present results implicate IL-1 and/or IL-6 as the major or perhaps the sole cytokines responsible for the total thymocyte co-mitogenic response observed in the CM assayed. This CM contains 1.1×10^4 u/ml of IL-6 (as assessed by the B9 cell assay) in the first point of the thymocyte proliferation curve shown in Fig. 1.F. According to our results with pure cytokines (Fig. 1.A) the concentration of IL-6 in the next dilution of CM should be inadequate to exert an effect over the IL-1-induced proliferation. In this case, only IL-1 must be exerting thymocyte co-mitogenic activity in this supernatant. This means that it is possible to measure IL-1 units specifically in this CM,

or in any IL-1-rich crude CM, such as those from LPS-induced monocytes.

Specificity for IL-1 measurement using the co-mitogenic assay would be enhanced by calculating units at greater dilutions on the dose-response curve, as already used by us to overcome inhibitory effects found in CM (S. R. Dalmau & C. S. Freitas, 1990a, *loc. cit.*). However, if non-stimulated or suboptimally stimulated cell supernatants are to be assayed, when the IL-6 secretion by human monocytes is supposed to be nearly its maximal (L. A. Aarden et al., 1987, *Eur. J. Immunol., 17:* 1411-1416), the IL-6:IL-1 ratio will be increased, and therefore the titration curves shall not be considered as measuring solely IL-1, even at high dilutions. In this latter situation, where the thymocyte co-mitogenic activity is poor, evaluation of the IL-1 or IL-6 contribution will require the addition of anti-human IL-6 blocking antibodies, as described before (M. Helle et al., 1988, *Eur. J. Immunol., 18:* 1535-1540).

Although the IL-1-driven, IL-6 synthesis from some target cells such as fibroblasts has been reported, suggesting an intermediary role for IL-6 in the IL-1 effect (J. A. Elias et al., 1989, *J. Immunol., 142:* 509-514; M. Helle et al., 1988, *Eur. J. Immunol., 18:* 957-959), the IL-6 amounts accumulated in the supernatants in their experiments (< 200 u/ml up to the third day of culture, with 1 x 10^5 thymocytes) do not seem to be enough to account for the co-stimulation observed. Furthermore, the endogenous IL-6 production does not appear to be an important pathway for IL-1 induction of murine bulk thymocyte proliferation: its effect (at 16 u/ml IL-1, plus PHA) could not be blocked by neutralizing amounts of anti-murine IL-6 antibodies (C. Uyttenhove et al., 1988, *J. Exp. Med., 167:* 1417-1427).

Materials and Acknowledgements — Recombinant human IL-1β (Hoffman-LaRoche, sp. act. 2x10^6 μ/mg protein), recombinant human TNFα (Hoffman-LaRoche, sp. act. 10^6 u/mg protein), and the TNF-sensitive WEHI 164 cell line were kindly donated by Dr Richard Peck, Basel Institute, SW. Recombinant human IL-6 (2.2x10^5 u/ml) was used as a supernatant of the P3X cell line (P. Poupart et al., 1987, *EMBO J., 6:* 1219-1224). It was kindly donated by Dr Una Chen, Basel Institute. PC61 hybridoma cells, were a kind gift of Dr Markus Nabholz, ISREC, Lausanne, SW. Hybridoma cell supernatant was used in a final dilution of 1:15.