HIGH AFFINITY BINDING OF $^{125}$I-LABELED HUMAN TUMOR NECROSIS FACTOR (LuKII) TO SPECIFIC CELL SURFACE RECEPTORS

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Tumor necrosis factor (TNF) was identified by Carswell et al. (1) during a study of the antitumor activity of serum from mice infected with bacillus Calmette-Guerin and subsequently injected with endotoxin. In a recent report (2), it was observed that human B cell lines produce a factor that is cytotoxic for mouse L cells sensitive to mouse TNF but not for L cells resistant to mouse TNF. The cytotoxic factor from one B cell line (LuKII) was partially purified and found to cause hemorrhagic necrosis of the Meth A mouse sarcoma in the standard in vivo mouse TNF assay (2). Based on these observations and other biological similarities to mouse TNF, this factor has been designated human TNF(LuKII).

TNF(LuKII) has now been purified to a specific activity of $1.5 \times 10^7$ U/mg of protein (3). Examination of this material by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) demonstrates the presence of seven protein bands with molecular weights ranging from 80,000 to 19,000 (3). The higher molecular weight forms are not aggregates of lower molecular weight components, as they cannot be dissociated by SDS, 2-mercaptoethanol, or urea. Peptide mapping analysis, as well as studies using a monoclonal antibody to TNF(LuKII), have demonstrated that all of the proteins present in these TNF preparations are related (3).

Many biologically active molecules, including hormones and lymphokines, have been found to bind to specific cell surface receptors. In the case of the interferons, it has been observed that resistance to the biological effects of an interferon can be due to a lack of appropriate cell surface receptors (4). Cell lines of human and mouse origin differ in their sensitivity to the cytotoxic/cytostatic effect of TNF (1, 5–7) and cells grown in the presence of TNF can become resistant to the effect of TNF (2). In this study, we demonstrate the existence of cell surface receptors to TNF and show that cells made resistant to TNF do not bind TNF(LuKII).

Materials and Methods

TNF Preparations and Assay. TNF(LuKII) was purified from culture fluids of the human LuKII lymphoblastoid cell line after induction with 10 ng/ml of mezerein (L. C. This work was supported by grants CA-38661 and CA-08748 from the National Cancer Institute.

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Services, Woburn, MA) (3). The specific activity of the TNF(LuKII) preparation was $1.5 \times 10^7$ U/mg of protein. Mouse serum TNF was purified by DEAE and G-100 Sephadex column chromatography and had a specific activity of $5 \times 10^4$ U/mg of protein. A more highly purified preparation of mouse TNF ($1 \times 10^8$ U/mg protein) was a gift of Dr. K. Haranaka.

TNF assays were performed in 96-well microtiter plates. TNF-sensitive L(M) cells were added at a density of $2 \times 10^4$ cells/well in 100 μl to fractions of TNF serially diluted twofold. After 2 d at 37°C, the plates were examined microscopically and the percentage of the killed cells was determined. The number of units of sample was calculated as the reciprocal of the highest dilution that killed 50% of the cells. All TNF assays were run in parallel with a TNF(LuKII) laboratory standard and all titers are expressed in laboratory units.

Radioiodination of TNF(LuKII). TNF(LuKII) was labeled with $^{125}$I using 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (Iodo-gen; Pierce Chemical Co., Rockford, IL) in the following manner. Polypropylene tubes were coated with 100 μg of Iodo-gen (dissolved in chloroform) by evaporation of the solvent. A sample of TNF(LuKII) containing 160,000 U/ml (as assayed in duplicate on three occasions) in 3 ml was incubated for 25 min at room temperature in an Iodo-gen-coated tube containing 6 mCi of $^{125}$I. The labeled protein was then separated from the unbound $^{125}$I using a P-4 column (Bio-Rad Laboratories, Richmond, CA) equilibrated with phosphate-buffered saline (PBS) containing 50 μg/ml of cytochrome c. The iodinated material eluted in the void volume of the column in 8 ml and was divided into aliquots before storing at $-80^\circ$C. It was assayed for biological activity six times on separate occasions and was found to contain 60,000 U/ml and $7.5 \times 10^9$ cpm/mg of TNF(LuKII). Thus, radioiodination of TNF(LuKII) did not result in any apparent loss of biological activity.

Assay for the Binding of $^{125}$I-labeled TNF(LuKII) to Cells. L(M) cells and TNF-resistant subclones of L(M) cells seeded in 25-cm² flasks at a density of $2 \times 10^6$ cells per flask were allowed to adhere to the flask for 24 h before exposure to $^{125}$I-labeled TNF(LuKII); at this time, the flask contained $\sim 3.5 \times 10^6$ cells per flask. HeLa cells seeded in 25-cm² flasks at a density of $1.5 \times 10^6$ cells per flask were allowed to adhere to the flask for 48 h before exposure to $^{125}$I-labeled TNF(LuKII); at this time, the flask contained $\sim 5 \times 10^6$ cells per flask. $^{125}$I-labeled TNF(LuKII) was added to the flask in 1 ml Eagle's minimum essential medium (MEM), containing 10% fetal bovine serum (Gibco Laboratories, Grand Island, NY). Amounts of TNF and time intervals are described in the figure legends. After the indicated incubation period, the cells were washed three times with cold PBS, scraped from the flask in PBS, and centrifuged at 15,000 g for 5 min over a cushion of PBS containing 5% sucrose. The sucrose cushion was then removed and the cell pellets counted in a gamma radiation counter. Specific binding of the radiolabeled TNF(LuKII) was determined by subtracting the number of counts bound to cells in the presence of an excess of nonlabeled TNF(LuKII) from the number of counts in the presence of $^{125}$I-labeled TNF(LuKII) alone. The amount of nonlabeled TNF(LuKII) used in each experiment is stated in the figure legend. Nonspecific binding of $^{125}$I-labeled TNF(LuKII) was not $\leq 15$ cpm in any experiment.

Results

Mouse L(M) cells and HeLa cells (a line derived from human cervical carcinoma) are sensitive to the cytotoxic effect of TNF(LuKII) and mouse TNF. L cells can be rendered resistant to TNF by repeated passage in TNF-containing medium, and two sublines of TNF-resistant L(M) cells have been developed: one made resistant to mouse TNF and the other, resistant to TNF(LuKII). Resistance to mouse TNF renders the cells resistant to TNF(LuKII), and vice versa.

As a first step, we examined the time course of binding of $^{125}$I-labeled TNF(LuKII) to the L(M) cells, L(M) cells resistant to TNF(LuKII), and HeLa cells. As shown in Fig. 1, the specific binding of $^{125}$I-labeled TNF(LuKII) to the
L(M) and HeLa cell lines occurred rapidly at 4°C and reached a steady state within 4 h. At 37°C, the amount of radiolabeled TNF(LuKII) bound to the cells continued to increase even after 8 h incubation. The amount of specific binding of 125I-labeled TNF(LuKII) is higher at 37°C than at 4°C. Comparable tests with L cells resistant to mouse TNF or TNF(LuKII) showed no specific 125I-labeled TNF(LuKII) binding even after 8 h exposure.

Quantitative competition experiments revealed that the binding of 125I-labeled TNF(LuKII) to L(M) or HeLa cells is equally inhibited by nonlabeled TNF(LuKII) and mouse TNF (Fig. 2).

To determine whether binding of TNF(LuKII) to the cells was occurring via a saturable receptor, we incubated L(M) and HeLa cells with increasing concentrations of 125I-labeled TNF(LuKII) for 5 h at 4°C and measured the uptake. Sites for TNF(LuKII) on L(M) and HeLa cell lines are half saturable at a TNF(LuKII) concentration of ~100 U/ml, demonstrating the presence of high affinity binding sites (Fig. 3). Three additional experiments were performed.
FIGURE 5. Scatchard analysis of the binding data depicted in Fig. 3. Specific binding was converted into Scatchard plots for the L(M) (A) and HeLa (B) cell lines.

(data not shown) and similar saturation curves were observed. Examination of the cytotoxic effect of TNF(LuKII) on L(M) and HeLa cells (Fig. 4) revealed that the receptor saturation curves and the dose response curves are roughly similar in their concentration dependence. Scatchard plots of the data in Fig. 3 are shown in Fig. 5. Based on TNF(LuKII) having a molecular weight, under nondenaturing conditions, of 70,000 (2, 3) and a specific activity of $1.5 \times 10^7$ U/mg of protein, we calculate the number of TNF receptors to be approximately 200 and 300 for the L(M) and HeLa cell lines, respectively, and the binding constant to be $\sim 1 \times 10^{-10}$ M for both L(M) and HeLa cells.

Discussion

Purified $^{125}$I-labeled TNF(LuKII) bound specifically to saturable high affinity receptors present on cell lines sensitive to the cytotoxic effect of TNF(LuKII) or mouse TNF. Cell lines rendered resistant to TNF appeared to lack specific receptors for TNF(LuKII). At 4°C, the binding of radiolabeled TNF(LuKII) to sensitive cells reached equilibrium after 4 h of exposure, while, at 37°C, the amount of TNF(LuKII) bound to the cells continued to increase over 8 h. These results suggest possible uptake or metabolism of the radiolabeled TNF(LuKII) at 37°C.

Mouse serum TNF competes with TNF(LuKII) for receptor binding on both mouse and human cell lines. Since the mouse TNF preparation used in these studies was not pure, the competition observed may be due to the contaminants. However, the observation that equal numbers of units of TNF(LuKII) and mouse TNF result in a similar degree of competition and that preparations of mouse TNF with different specific activities compete to an equivalent extent (data not shown) suggest that mouse TNF molecules are competing with TNF(LuKII) for receptor binding. It will be important to carry out a similar receptor studies with recombinant human TNF (8–10) and nonrecombinant and recombinant human lymphotoxin (11, 12) to determine whether a common receptor exists for these various cytotoxic factors.

Under denaturing conditions, TNF(LuKII) is observed to exist in molecular weight forms ranging from 80,000 to 19,000 (3) and, under nondenaturing conditions, as a 70,000 mol wt molecule. We have used this latter value to calculate the number of receptors per cell and the binding constant of the receptor. The number of receptors would be higher if we calculated on the basis of the lower molecular weight forms of TNF(LuKII).
Williamson et al. (2) have defined the TNF(LuKII) sensitivity of a large panel of human cell lines, and studies are currently underway to determine whether TNF response correlates with the presence and number of TNF receptors. If so, tests for TNF receptors on tumor biopsies may be of predictive value in determining the TNF sensitivity of individual tumors.

Summary

125I-labeled TNF(LuKII) (tumor necrosis factor) binds specifically to human and mouse cell lines sensitive to the cytotoxic effect of TNF, but not to cells made resistant to TNF. TNF-sensitive cells have cell surface receptors with a high affinity for TNF(LuKII). Mouse TNF competes with TNF(LuKII) for receptor binding. Scatchard analysis of the binding data yielded linear plots and suggests that TNF(LuKII) binds to homogeneous receptor sites. The number of TNF(LuKII) receptors on two TNF-sensitive cell lines is 200–300 per cell and the affinity constant of the receptor for TNF(LuKII) is ~1 x 10^{-10} M.

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Note added in proof: Recombinant human TNF provided by Dr. Michael Pallidino, Genentech, Inc., competes with TNF(LuKII) for TNF receptor binding.

References

1. Carswell, E. A., L. J. Old, R. L. Kassel, S. Green, N. Fiore, and B. Williamson. 1975. An endotoxin-induced serum factor that causes necrosis of tumors. Proc. Natl. Acad. Sci. USA. 25:3666.
2. Williamson, B. D., E. A. Carswell, B. Y. Rubin, J. S. Prendergast, and L. J. Old. 1983. Human tumor necrosis factor produced by human B-cell lines: synergistic cytotoxic interaction with human interferon. Proc. Natl. Acad. Sci. USA. 80:5397.
3. Rubin, B. Y., S. L. Anderson, S. A. Sullivan, B. D. Williamson, E. A. Carswell, and L. J. Old. 1985. Purification and characterization of a human tumor necrosis factor. Proc. Natl. Acad. Sci. USA. In press.
4. Aguet, M. 1980. High affinity binding of 125I-labeled mouse interferon to a specific cell surface receptor. Nature (Lond.). 284:459.
5. Helson, L., S. Green, E. Carswell, and L. J. Old. 1975. Effect of tumor necrosis factor on cultured human melanoma cells. Nature (Lond.). 258:731.
6. Haranaka, K., and N. Satomi. 1981. Cytotoxic activity of tumor necrosis factor (TNF) on the human cancer cells in vitro. Jpn. J. Exp. Med. 51:191.
7. Green, S., A. Dobrjansky, M. A. Chiasson, E. Carswell, M. K. Schwartz, and L. J. Old. 1977. Corynebacterium parvum as the priming agent in the production of tumor necrosis factor in the mouse. J. Natl. Cancer Inst. 59:1519.
8. Pennica, D., G. E. Nodwin, J. S. Hayflick, P. H. Seeburg, R. Derynck, M. A. Palladino, W. J. Kohr, B. B. Aggarwal, and D. V. Goeddel. 1984. Human tumor necrosis factor: precursor structure, expression and homology to lymphotoxin. Nature (Lond.). 312:724.
9. Shirai, T., H. Yamaguchi, H. Ito, C. W. Todd, and R. B. Wallace. 1985. Cloning and expression in Escherichia coli of the gene for human tumor necrosis factor. Nature (Lond.). 313:803.
10. Wang, A. M., A. A. Creasey, M. B. Ladner, L. S. Lin, J. Strickler, J. N. Van Arsdell,
R. Yamamoto, and D. F. Mark. 1985. Molecular cloning of the complementary DNA for human tumor necrosis factor. *Science (Wash. DC).* 228:149.

11. Granger, G. A., R. S. Yamamoto, D. S. Fair, and J. C. Hiserodt. 1978. The human LT system. I. Physical-chemical heterogeneity of LT molecules released by mitogen-activated human lymphocytes in vitro. *Cell. Immunol.* 33:388.

12. Gray, P. W., B. B. Aggarwal, C. V. Benton, T. S. Bringman, W. J. Henzel, J. A. Jarrett, D. W. Leung, B. Moffat, P. Ng, L. P. Sverdinsky, M. A. Palladino, and G. E. Nedwin. 1984. Cloning and expression of cDNA for human lymphotxin, a lymphokine with tumor necrosis activity. *Nature (Lond.)* 312:721.