A Newly Defined Property of Somatotropin: Priming of Macrophages for Production of Superoxide Anion

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Macrophages can be activated to produce reactive oxygen intermediates, such as superoxide anion (O_2^-), which are responsible for intracellular killing of pathogenic microbes. Treatment with either native or recombinant somatotropin augmented the production of O_2^- by both peripheral blood–derived and alveolar macrophages stimulated with opsonized zymosan in vitro. This effect was abolished by prior treatment with an antibody specific for somatotropin. When either native or recombinant porcine somatotropin or native rat somatotropin was administered to hypophysectomized rats in vivo, activation of peritoneal macrophages, as measured by release of O_2^- in response to opsonized zymosan, was equivalent to that of macrophages from rats primed with the macrophage-activating factor interferon-γ. Priming of macrophages in vivo was observed at physiologically relevant doses of somatotropin that caused a 10 to 40 percent increase in growth rate. Priming of mononuclear phagocytes for augmented production of reactive oxygen metabolites is a newly defined property of somatotropin.

MACROPHAGES THAT HAVE BEEN infected with an intracellular pathogen acquire augmented capacity to kill bacteria (*1*). Macrophage priming and activation are accompanied by enhanced respiratory burst activity and secretion of reactive oxygen intermediates, such as hydroxyl radicals, singlet oxygen molecules, and superoxide anion (O_2^-). These reactive oxygen metabolites are the most important oxygen-dependent pathway by which macrophages kill ingested microbes (*2*). Interferon-γ (IFN-γ), one of the factors produced by antigen- or mitogen-activated T cells, serves to prime macrophages for increased production of reactive oxygen metabolites (*3, 4*). Diseases in which production of IFN-γ is diminished, such as acquired immunodeficiency syndrome (*5*), leprosy (*6*), and visceral leishmaniasis (*7*), often lead to overwhelming opportunistic infections.

Although IFN-γ has been identified as the primary T cell product that activates macrophages for enhanced O_2^- production (*4*), other endogenous substances that augment respiratory burst activity probably exist (*8*). A recent study demonstrated that proliferating spleen cells express messenger RNA for somatotropin (*9*), suggesting that somatotropin might function as a cytokine. Although it is well known that somatotropin is secreted by the adenohypophysis and participates in a number of growth processes, its role in immunoregulation has not been thoroughly explored. We recently reported that somatotropin, as well as GH_3 pituitary adenoma cells, which secrete both somatotropin and prolactin, restore the T cell proliferative responses and interleukin-2 (IL-2) synthesis that are naturally diminished in aged rats (*10*). Somatotropin also augments the activity of cytolytic T cells (*11*), natural killer cells (*10, 12*), and delayed-type hypersensitivity T cells (*13*), as well as antibody synthesis in response to T-dependent antigens (*13*) and production of the thymic hormone thymulin (*14*).

During our studies on somatotropin, we incubated porcine liver cell suspensions with somatotropin and noticed a marked change in the morphology of Kupffer cells that was characteristic of activated macrophages. Because activated macrophages serve several functions in the immune response (*15*), we postulated that another physiological role of somatotropin could be to augment responses of macrophages. We now report that the pituitary hormone somatotropin primes mononuclear phagocytes for augmented production of O_2^-.

To test the hypothesis that somatotropin can augment O_2^- production, we measured O_2^- release from somatotropin-treated macrophages in response to opsonized zymosan by reduction of ferricytochrome c with the concomitant increase in absorbance of light at 550 nm. Native pituitary-derived porcine somatotropin (npST) was incubated for 24 hours with porcine blood–derived mononuclear phagocytes, and O_2^- concentrations were measured. In a typical experiment (Fig. 1), normal mononuclear cells released only small amounts of O_2^- (36 nmol per milligram of protein per hour) when stimulated with opsonized zymosan. Lipopolysaccharide (LPS), which is a potent inducer of O_2^- (*16*) and which served as our positive control in this experiment, caused the release of 228 nmol of O_2^- per milligram of protein per hour when cells were incubated with opsonized zymosan. When mononuclear phagocytes were incubated with npST and then stimulated with opsonized zymosan, the dose-response curve yielded significant linear (mean ± SEM, 6.9 ± 2.1, $P < 0.01$) and quadratic (−0.006 ± 0.002, $P < 0.03$) components. This positive effect was not caused by contaminating hormones in the npST because recombinant porcine somatotropin (rpST) yielded an equivalent dose-response curve with similar linear (9.0 ± 1.2, $P < 0.01$) and quadratic (−0.008 ± 0.001, $P < 0.01$) slopes. Furthermore, the 18-fold increase in O_2^- production that was caused by overnight incubation with npST (500 ng/ml) was reduced by 90% when mononuclear phagocytes were previously incubated with a specific heat-inactivated guinea pig antibody (1/8000 dilution) to somatotropin (*17*). When rpST (500 ng/ml) was used, there was the same increase

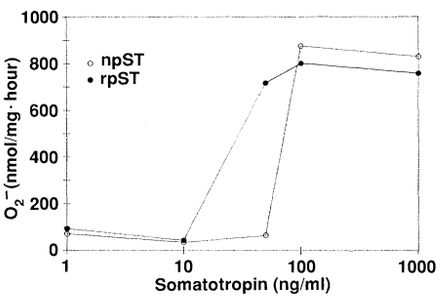

Fig. 1. Native, pituitary-derived porcine somatotropin (npST) and recombinant porcine somatotropin (rpST) augment the production of O_2^- in vitro by porcine peripheral blood–derived mononuclear phagocytes. Results from one of four experiments are shown in which peripheral blood was collected from 10-week-old pigs and monocyte-derived mononuclear phagocytes were separated on a plasma Percoll gradient (*25*). Adherent cells (>90% positive for α-naphthyl butyrate esterase) were prepared and incubated in Dulbecco's modified Eagle's medium with 2% heat-inactivated fetal bovine serum (Hyclone, Sterile Systems, Logan, Utah) containing either LPS [10 endotoxin units (E.U.) per milliliter; 5 E.U. is equivalent to 1 ng of LPS; M. A. Bioproducts, Walkersville, Maryland] as a positive control or various concentrations of npST or rpST. All tissue culture reagents were free of endotoxin (assay sensitivity = 0.01 E.U./ml) as assessed by the chromogenic *Limulus* amoebocyte lysate assay (M. A. Bioproducts). After a 24-hour incubation with somatotropin, opsonized zymosan (1 mg/ml; Packard, Chicago, Illinois) was added and the incubation was continued for 4 hours. Superoxide anion was determined in triplicate samples, as described (*18, 23*).

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in respiratory burst activity relative to that of control cells, and the specific somatotropin antibody totally blocked (102%) the enhancement in O_2^- production. Normal heat-inactivated guinea pig serum did not affect the production of O_2^-.

Porcine alveolar macrophages (>98% stained positive with α-naphthyl butyrate esterase) were then used to determine if somatotropin could substitute for IFN-γ in enhancing O_2^- production by a purified population of macrophages. Opsonized zymosan stimulated the release of 199 nmol of O_2^- per milligram of protein per hour from alveolar macrophages, and this effect was totally blocked by superoxide dismutase (Table 1). The amount of O_2^- released by both npST- and rpST-treated macrophages in response to opsonized zymosan was more than twice the O_2^- released by control cells. Furthermore, the enhancing effect of rpST was totally blocked by an antiserum specific for somatotropin. Therefore, it appears that somatotropin directly primes macrophages for augmented O_2^- production rather than stimulating IFN-γ production by contaminating T lymphocytes, although we have not yet tested the synergistic effects of somatotropin and IFN-γ.

These data provided evidence that somatotropin affects macrophages by augmenting the production of O_2^- in vitro. We therefore asked whether somatotropin would also prime macrophages for enhanced O_2^- production in vivo. To conduct these experiments, we used hypophysectomized rats. Since the pituitary source of somatotropin was removed in these rats, confounding effects with endogenous somatotropin in plasma were avoided. Hypophysectomized Sprague-Dawley rats were injected once daily with various concentrations of npST, rpST, or native, pituitary-derived rat somatotropin (nrST) or with vehicle alone for nine consecutive days. An optimal concentration of recombinant rat IFN-γ (*18*), which is a potent inducer of O_2^- production both in vitro and in vivo (*3*), was used as a positive control. An adequate number of cells for the subsequent analysis of O_2^- was obtained by pooling peritoneal cells from two rats. As expected, npST, rpST, and nrST caused significant increases in growth rate, ranging from 10% to 40% during the 10-day growth period (Table 2). Macrophages from the two control groups (hypophysectomized-untreated and hypophysectomized–vehicle-treated) released no superoxide dismutase–inhibitable O_2^- (Table 2). In contrast, macrophages from hypophysectomized rats given recombinant rat IFN-γ released 417 nmol of O_2^- per milligram of protein per hour when stimulated with opsonized zymosan. Hypophysectomized rats injected daily for 9 days with 12 μg of npST released 268 nmol of O_2^- per milligram of protein per hour, and even greater amounts (438 nmol of O_2^- per milligram of protein per hour) were released from macrophages of rats treated with 24 μg of npST. Both these values were significantly greater than values obtained with the two negative controls. The rpST at both 12 and 24 μg also induced significant increases in the production of O_2^-, and the magnitude of the increase was equivalent to that induced by identical concentrations of npST. Priming of rat peritoneal macrophages for augmented production of O_2^- by somatotropin was not simply caused by injection of exogenous foreign protein because nrST also caused a significant augmentation in the production of O_2^-.

A number of exogenous macrophage-priming factors, such as LPS (*16*), muramyl dipeptide (*16, 18*), and polyinosinate: polycytidylate (*19*), augment the generation of oxygen radicals by macrophages. To date, however, IFN-γ has been the only completely characterized substance known to be produced by mammalian cells that can prime macrophages to produce enhanced quantities of O_2^- (*3*). Somatotropin, which is synthesized by the adenohypophysis, may also be produced by activated leukocytes (*9*). Our data demonstrate that somatotropin, administered in either native or recombinant forms and tested in both in vitro and in vivo systems, augments the production of O_2^- by mononuclear phagocytes. When ad-

Table 1. Priming of porcine alveolar macrophages in vitro by native pituitary-derived porcine somatotropin (npST) and recombinant porcine somatotropin (rpST). Alveolar macrophages (>98% positive for α-naphthyl butyrate esterase) from eight 10-day-old female pigs were obtained by pulmonary lavage and isolated by using published methodologies (*22*). Cells were treated with hormones for 24 hours and then assayed for O_2^- in response to opsonized zymosan (op zym) (*18, 23*). Analysis of variance revealed significant treatment effects ($F = 16.2$; 6, 27 df; $P < 0.001$). Means with different superscripts are different ($P < 0.05$). Differences among means were detected with Duncan's new multiple range test (*24*).

Treatment	O_2^- (nmol/mg · hour)	SEM
Unstimulated	28*	14
Stimulated with op zym	199†	48
Op zym + superoxide dismutase	28*	9
Op zym + npST (500 ng/ml)	430‡	90
Op zym + rpST (500 ng/ml)	431‡	81
Op zym + rpST + ST antibody	48*	20
Op zym + ST antibody	141*,†	30

Table 2. Native pituitary-derived porcine somatotropin (npST), recombinant porcine somatotropin (rpST), and native, pituitary-derived rat somatotropin (nrST) induce respiratory burst activity in rat peritoneal macrophages in vivo. Six-week-old albino female Sprague-Dawley rats (Johnson Laboratories, Bridgeview, Illinois) that had been hypophysectomized at least 15 days earlier were injected subcutaneously each day with recombinant rat IFN-γ [500 units, specific activity = 4×10^6 units per milligram of protein (when tested on rat RATEC cells); AMGEN, La Jolla, California], rpST, npST, or nrST in 200 μl of a mixture of 0.15*M* NaCl and 0.03*M* $NaHCO_3$, *p*H 9.5, for 9 days. Complete hypophysectomy was confirmed by both gross and histological examination [4-μm sections that were stained with hematoxylin and eosin (*10*)] at the end of the experiment. Body weight was measured and resident peritoneal macrophages were collected (*18, 23*) 24 hours after the last injection of hormone. One-way analysis of variance was used to analyze results of daily weight gain ($F = 184.1$; 12, 69 df; $P < 0.001$) and O_2^- production induced by opsonized zymosan that was inhibitable by superoxide dismutase ($F = 13.6$; 12, 69 df; $P < 0.001$), and differences among means were detected by Duncan's new multiple range test (*24*). Means within a column with different superscripts are different ($P < 0.01$).

Item	*n*	Dose/day	Growth (g/day)		O_2^- (nmol/mg · hour)	
			Mean	SEM	Mean	SEM
Hypophysectomized rats	6		0.392*	0.08	−40*	20
+ Vehicle	11	200 μl	0.282*	0.05	−17*	7
+ Rat IFN-γ	11	500 units	0.252*	0.06	417†	79
+ npST	5	6 μg	1.322‡	0.09	0*	19
	6	12 μg	1.633§	0.07	268†	71
	5	24 μg	2.060‖,¶	0.05	438†	70
+ rpST	6	6 μg	0.815†	0.03	−62*	14
	6	12 μg	1.067†,‡	0.07	280†	59
	6	24 μg	1.297‡	0.07	344†	42
+ nrST	5	12 μg	1.850§,‖	0.11	3*	4
	5	24 μg	2.320¶	0.10	267†	31
	5	48 μg	2.870#	0.10	247†	16
	5	96 μg	3.440**	0.10	309†	22

ministered in vivo, somatotropin induces amounts of O_2^- similar to those produced by IFN-γ at a concentration that is optimal for macrophage activation (*18*). Thus, somatotropin shares at least one biologic activity with IFN-γ, priming macrophages to produce augmented amounts of O_2^-. It will be important to learn whether other macrophage-activating properties of IFN-γ are also shared by somatotropin.

These data expand earlier results that have shown that somatotropin is involved in the regulation of immune events in vivo (*10–14*). Macrophages are central to the induction and expression of many immune responses, so perhaps a fundamental mechanism of action of somatotropin is at the level of macrophages. Since we used animals that had their pituitary source of somatotropin removed, these results may be particularly important for growth hormone–deficient children who receive exogenous recombinant somatotropin to stimulate growth. Although excessive amounts of reactive oxygen metabolites can damage host tissues and can kill intracellular bacteria (*20*), we believe that this discovery of macrophages as one target for the action of somatotropin is important for understanding reciprocal relationships between the immune and endocrine systems (*21*).

REFERENCES AND NOTES

1. G. B. Mackaness, *J. Exp. Med.* **129**, 973 (1969); D. O. Adams and T. A. Hamilton, *Annu. Rev. Immunol.* **2**, 283 (1984).
2. B. M. Babior, *N. Engl. J. Med.* **298**, 659 (1978); R. B. Johnston, Jr., *Lymphokines* **3**, 33 (1981); C. F. Nathan, M. I. Karnovsky, J. R. David, *J. Exp. Med.* **133**, 1356 (1971).
3. C. F. Nathan, H. W. Murray, M. E. Wiebe, B. Y. Rubin, *J. Exp. Med.* **158**, 670 (1983); H. W. Murray, G. Spitalny, C. F. Nathan, *J. Immunol.* **134**, 1619 (1985); C. M. Black, J. R. Catterall, J. S. Remington, *J. Immunol.* **138**, 491 (1987).
4. C. F. Nathan *et al.*, *J. Exp. Med.* **160**, 600 (1984).
5. H. W. Murray *et al.*, *N. Engl. J. Med.* **310**, 883 (1984); H. C. Lane *et al.*, *ibid.* **313**, 79 (1985); H. W. Murray *et al.*, *ibid.*, p. 1504; H. W. Murray *et al.*, *J. Immunol.* **138**, 2457 (1987).
6. N. Nogueira *et al.*, *J. Exp. Med.* **158**, 2165 (1983); C. F. Nathan *et al.*, *N. Engl. J. Med.* **315**, 6 (1986).
7. E. M. Carvalho, R. Badaró, S. G. Reed, T. C. Jones, W. D. Johnson, Jr., *J. Clin. Invest.* **76**, 2066 (1985); C. F. Nathan *et al.*, *J. Immunol.* **138**, 2290 (1987).
8. M. S. Cohen, D. E. Mesler, R. G. Snipes, T. K. Gray, *J. Immunol.* **136**, 1049 (1986); E. J. Wing, N. M. Ampel, A. Waheed, R. K. Shadduck, *ibid.* **135**, 2052 (1985); R. D. Schreiber, L. J. Hicks, A. Celada, N. A. Buchmeier, P. W. Gray, *ibid.* **134**, 1609 (1985); C. A. Nacy, A. H. Fortier, M. S. Meltzer, N. A. Buchmeier, R. D. Schreiber, *ibid.* **135**, 3505 (1985); P. H. Krammer, C. F. Kubelka, W. Falk, A. Ruppel, *ibid.*, p. 3258; H. P. Hartung and K. V. Toyka, *Eur. J. Pharmacol.* **89**, 301 (1983).
9. P. C. Hiestand *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2599 (1986).
10. K. W. Kelley *et al.*, *ibid.*, p. 5663; D. R. Davila *et al.*, *J. Neurosci. Res.* **18**, 108 (1987).
11. C. Snow, T. L. Feldbush, J. A. Oaks, *J. Immunol.* **126**, 161 (1981).
12. W. Kiess, H. Doerr, E. Eisl, O. Butenandt, B. H. Belohradsky, *N. Engl. J. Med.* **314**, 321 (1986); O. B. Saxena, R. K. Saxena, W. H. Adler, *Int. Arch. Allergy Appl. Immunol.* 67, 169 (1982).
13. E. Nagy, H. G. Friesen, A. H. Sehan, I. Berczi, *Endocrinology* **116**, 1117 (1985); W. Pierpaoli, C. Baroni, N. Fabris, E. Sorkin, *Immunology* **16**, 217 (1969); N. Fabris, W. Pierpaoli, E. Sorkin, *Clin. Exp. Immunol.* **9**, 227 (1971).
14. B. L. Goff, J. A. Roth, L. H. Arp, G. S. Incefy, *Clin. Exp. Immunol.* **68**, 580 (1987); N. Fabris, E. Mocchegiani, P. Paolucci, A. Balsamo, E. Cacciari, *Clin. Exp. Immunol.*, in press.
15. E. R. Unanue and P. M. Allen, *Science* **236**, 551 (1987); S. E. Loveless and G. H. Heppner, *J. Immunol.* **131**, 2074 (1983); K. H. Mahoney, A. M. Fulton, G. H. Heppner, *ibid.*, p. 2079.
16. M. J. Pabst and R. B. Johnston, Jr., *J. Exp. Med.* **151**, 101 (1980).
17. D. N. Marple and E. D. Aberle, *J. Anim. Sci.* **34**, 261 (1972).
18. C. K. Edwards III *et al.*, *J. Immunol.* **136**, 1820 (1986).
19. A. M. Badger, *J. Leukocyte Biol.* **40**, 725 (1986); S. J. Leibovich and R. Ross, *Am. J. Pathol.* **78**, 71 (1975).
20. B. Halliwell, *FASEB* **1**, 358 (1987).
21. J. E. Blalock, *Lymphokines* **9**, 1 (1984); K. W. Kelley, *J. Anim. Sci.*, in press.
22. R. L. McGuire and L. A. Babiuk, *J. Reticul. Soc.* **31**, 251 (1982); B. Charley, *Ann. Rech. Vet.* **13**, 1 (1982); H. Bielefeldt Ohmann and L. A. Babiuk, *J. Leukocyte Biol.* **39**, 167 (1986); F. Y. El-Awar and E. C. Hahn, *Am. J. Vet. Res.* **48**, 481 (1987); D. O. Slauson, J. C. Lay, W. L. Castleman, N. R. Neilsen, *ibid.* **41**, 412 (1987).
23. R. B. Johnston, C. A. Godzik, Z. A. Cohn, *J. Exp. Med.* **148**, 115 (1978); P. R. Gangadharam and C. K. Edwards III, *Am. Rev. Respir. Dis.* **130**, 834 (1984).
24. *SAS User's Guide: Statistics* (SAS Institute, Cary, NC, 1985).
25. C. Haslett, L. A. Guthrie, M. M. Kopaniak, R. B. Johnston, Jr., P. M. Henson, *Am. J. Pathol.* **119**, 101 (1985); R. A. Musson, *ibid.* **111**, 331 (1983).

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Subset-Specific Expression of Potassium Channels in Developing Murine T Lymphocytes

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Ion channels were studied in four major subsets of developing murine thymocytes by using patch-clamp recording and cell-surface staining techniques. The expression of three types of voltage-gated potassium channels in thymocytes varies consistently with the cell's developmental state and functional class as defined by cell-surface markers. One class of potassium channel (type *n*) predominates in immature thymocyte subsets as well as in mature-phenotype $CD4^+CD8^-$ thymocytes (precursors to helper T lymphocytes), and the average surface density of this channel type correlates with the extent of cell proliferation. Two additional types of potassium channels (types *n'* and *l*) are found in the mature $CD4^-CD8^+$ thymocyte subset that contains precursors to cytotoxic and suppressor T cells. The subset-specific expression of type *n'* and *l* potassium channels suggests their use as additional cell-surface markers with which to identify precursors to the cytotoxic suppressor T cell lineage.

THE MOST PREVALENT VOLTAGE-GATED channels in mature T lymphocytes are potassium channels, similar in several respects to delayed rectifier channels of nerve and muscle (*1–5*). At least two distinct types of voltage-gated K^+ channels have been observed in T lymphocytes from mice (*5, 6*), but their developmental origins and distribution among the several functional classes of T cells have not been determined. To investigate this question, we have applied patch-clamp recording techniques (*7*) to subsets of developing T cells in the murine thymus. Thymocyte subsets were identified by staining with fluorescently labeled monoclonal antibodies to CD4 (L3T4) and CD8 (Lyt-2) membrane glycoproteins or with fluorescently labeled peanut agglutinin (PNA). Functionally immature thymocytes are generally $CD4^-CD8^-$ (double-negative) or $CD4^+CD8^+$ (double-positive; also PNA^+), whereas the phenotypes of the remaining thymocytes are the same as those of mature T cells; that is, $CD4^+CD8^-$ or $CD4^-CD8^+$ (*8–10*). After phenotypic identification by epifluorescence microscopy (*11*) (see cover), each cell was voltage-clamped in the whole-cell or excised-patch recording mode (*7*) to characterize the channels present in its membrane.

In whole-cell recordings from over 200 thymocytes, we observed three types of voltage-dependent K^+ channels, termed *n*, *n'*, and *l*. A set of biophysical and pharmacological properties provides a "fingerprint" for identifying each of the three classes of K^+

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