perfusions originated from activation of plasma factors or from contamination by "tissue thromboplastin" has been explored. The assay method for factor VIII was insensitive to the addition of active factor I (activated Christmas factor-PTC′) and active factor XI (activation product); it was sensitive to "tissue thromboplastin" only in concentrations greater than 1 : 10, concentrations greatly in excess of those that might occur physiologically. The activity of factor VIII perfusate when generated with human hemophilic plasma priming volumes was destroyed by heating to 56°C for 10 minutes. These observations supported the validity of this two-stage assay as representing factor VIII in the perfusates rather than "tissue thromboplastin" or the products of early-stage coagulation reactions. The standard reference plasma, assigned an arbitrary level of 100 percent, was commercial lyophilized plasma (*11*) prepared from 50 or more donors.

The results of 35 studies are summarized in Tables 1 and 2. Before each perfusion, no AHF activity could be detected in the effluent after the specimens were flushed. In control perfusions of both liver and spleen, without specimens in the circuits, AHF activity progressively decreased. Pig livers perfused with homologous blood produced overall increases in AHF activity of the perfusate. Livers perfused with residual blood from open-heart surgical procedures and with fresh heterologous (human) priming volumes produced increases and decreases in AHF activity, respectively.

Perfusion of spleens with autologous, homologous, and heterologous (human) blood resulted in decreases in AHF activity of the perfusate. However, pig spleens perfused with heterologous (human) AHF-deficient priming volumes resulted in progressive increases in AHF activity of the perfusate, 0.7 to 12 times baseline determinations. A representative experiment with pig spleen and AHF-deficient perfusate is shown in Fig. 1. Thus, the liver and spleen are sites of release of AHF activity by synthesis or storage mechanisms, and the targets of an AHF-stimulating substance in the plasma of hemophiliacs.

Splenic homotransplantation is feasible. Five human allografts have been undertaken. After operation in one, a child with hypogammaglobulinema, evidence of transplanted splenic function was present, as indicated by a postoperative increase in serum γ-globulin (*12*). The application of whole-organ transplantation of a permanent AHF source in hemophilia is suggested. The spleen, as indicated by the data and the technical ease of procurement and transplantation, would seem suitable for this purpose.

JOHN C. NORMAN
JEAN-PIERRE LAMBILLIOTTE
YASUSHI KOJIMA, HERBERT S. SISE
Departments of Surgery and *Medicine, Harvard Medical School* and *Tufts University Medical School, Boston City Hospital, Boston, Massachusetts*

References and Notes

1. R. Biggs and R. G. MacFarlane, Eds. *Treatment of Haemophilia and Other Coagulation Disorders* (Davis, Philadelphia, 1966), pp. 43 and 81.
2. R. G. MacFarlane, in *The Plasma Proteins*, F. W. Putnam, Ed. (Academic Press, New York, 1960), vol. 2. p. 173.
3. H. S. Sise, J. F. Newcomb, J. C. Norman, W. V. McDermott, Jr., *Fed. Proc.* **25**, 620 (1966).
4. J. G. Pool and T. H. Spaet, *Proc. Soc. Exp. Biol. Med.* **87**, 54 (1954).
5. R. A. Weaver, R. E. Price, R. D. Langdell, *Amer. J. Physiol.* **206**, 335 (1964).
6. E. P. Libre, D. H. Cowan, N. R. Shulman, *Blood* **26**, 890 (1965).
7. J. G. Pool, *Fed. Proc.* **25**, 317 (1966).
8. W. P. Webster, R. L. Reddick, H. R. Roberts, G. D. Penick, *Nature* **213**, 1146 (1967); W. P. Webster and G. D. Penick, *Fed. Proc.* **26**, 487 (1967).
9. J. C. Norman, C. A. Saravis, M. E. Brown, W. V. McDermott, *Surgery* **60**, 179 (1966).
10. J. G. Pool and J. Robinson, *Brit. J. Haematol.* **5**, 17 (1959).
11. Normal plasma control, Baltimore Biological Laboratories, Baltimore, Maryland.
12. T. L. Marchioro, T. E. Starzl, W. R. Waddell, *Ann. N.Y. Acad. Sci.* **120**, 626 (1964).
13. Supported by grants AM 10517, AM 08681 and HE 10056 from U.S. Public Health Service. J.C.N. is a Markle Scholar in Academic Medicine. We thank Martha J. Cook, Rose Langer, Linda Levine, Jutta Booth, J. E. Martin and W. J. Robinson for technical assistance.

21 August 1967

Murine Lymphoma: Augmented Growth in Mice with Pertussis Vaccine–Induced Lymphocytosis

Abstract. *Injection of* Bordetella pertussis *vaccine caused an excessive lymphocytosis, associated with an augmented growth of a lymphoma cell homotransplant in mice. A markedly impaired reactive cell proliferation was revealed in the spleens of the vaccine-pretreated mice after stimulation with phytohemagglutinin or Freund's complete adjuvant.*

Lymphocytes play a central role in immune response, especially in homograft rejection (*1*). Lymphopenia can be induced in animals by various procedures, namely, x-irradiation (*2*), neonatal thymectomy (*3*), cortisone treatment (*4*), thoracic duct drainage (*5*), and others. Animals rendered lymphopenic by these methods become immunologically deficient. This deficiency manifests itself not only by delayed homograft rejection (*5*, *6*) but also by enhanced carcinogenesis, viral (*7*) or chemical (*8*), and also, possibly, by enhanced growth of transplanted tumors.

A study was performed to investigate the effect of lymphocytosis on the growth of a tumor homograft. The tumor used in this experiment is an undifferentiated lymphoma induced in a female Swiss mouse inoculated with a long-term tissue culture line of the Rauscher leukemia virus (*9*). All mice used derive from a random-bred Swiss line of the Texas Inbred Mouse Company, Houston.

An extreme lymphocytosis ranging from 50,000 to 118,000 per cubic millimeter was induced in 3- to 4-week-old mice by injecting 0.3 ml of commercially available *Bordetella pertussis* vaccine (BPV) intravenously. Control mice received 0.3 ml of saline intravenously. Subcutaneous tumor inoculations were made by injecting 5000 viable lymphoma cells in the scapular area. Periodic observation was then made of the occurrence of palpable tumors and their growth; tumor size was recorded as an average of the longest diameter and the one crossing it at right angles.

It appears that the tumor is antigenic in these mice and the "take" rate of the inoculum consisting of 5000 viable cells is 50 percent in mice of the age group used. At least one antigenic marker could be attributed to leukemia virus antigens (*10*).

In the first set of experiments, lymphoma cells were inoculated at the time of maximal lymphocytosis, that is, 4 days after BPV injection. Figure 1 (left) shows the results of a typical experiment. An augmented tumor growth was manifested by earlier appearance of measurable tumors and a larger tumor size on each measurement, as compared to the control group. In a total of four such experiments, using 32 mice in the pretreated and 28 mice in the control groups, it

was clearly shown that groups with lymphocytosis had a higher "take" rate (90 percent versus 55 percent in controls).

In the second set of experiments, BPV was injected on the 7th day after the inoculation of tumor. In this case, difference in tumor growth between groups with lymphocytosis and controls occurred about 14 to 17 days after inoculation of tumor (Fig. 1, right). In control mice a defense mechanism became operative, decelerating the rate of tumor growth, while in mice with lymphocytosis, tumors continued to grow at the same rate. In two experiments using 18 mice in the treated and 16 mice in the control groups, the overall "take" rate was also higher in groups with lymphocytosis (83 percent versus 64 percent in controls). More significantly, autopsy findings indicate that the spread of the tumor from the initial site was also more advanced in the experimental than in the control group in both sets of experiments.

These results show that mice with BPV-induced lymphocytosis may not possess an effective immune response, in spite of very large numbers of circulating lymphocytes. In order to further substantiate this hypothesis, reactivity of the BPV-treated mice to phytohemagglutinin-M and Freund's complete adjuvant (FA) was studied.

Mice treated with BPV were injected at the time of maximal lymphocytosis with 1 ml of diluted PHA solution intravenously or 0.5 ml of a mixture of FA and saline intraperitoneally. Erythroagglutinin had been removed from phytohemagglutinin-M by repeated adsorption onto human red cells. Control groups included normal mice as well as those treated with one of the three agents alone. Four days later mice were killed and their spleens were examined histologically. The appearance of pyroninophilic cells, the size and structure of the Malpighian follicles, the width of red pulp between follicles, and the number of nucleated cells within the red pulp were compared. Normal spleens showed moderate cellular activity within the germinal centers, a good supply of small lymphoid cells, and a moderate number of nucleated cells within the red pulp (Fig. 2A). Depletion of small lymphocytes in lymphoid follicles, little activity in the germinal centers, and decreased number of nucleated cells in the red pulp were marked in the BPV-treated spleen (Fig. 2B). Phytohemagglutinin-M caused an emergence of large "blast" cells in the white as well as the red pulp; the lymphoid follicles enlarged and often coalesced, and the red pulp was filled with nucleated cells (Fig. 2C).

Phytohemagglutinin-M failed to cause any noticeable cell proliferations in the spleens of BPV-pretreated mice (Fig. 2D), resulting in a pattern identical to that seen after BPV-treatment alone. After FA injection, reticulum cell proliferation in the white pulp and enlargement of the germinal centers with active phagocytosis were evident; FA also failed to stimulate cell proliferation in spleens of BPV-pretreated mice.

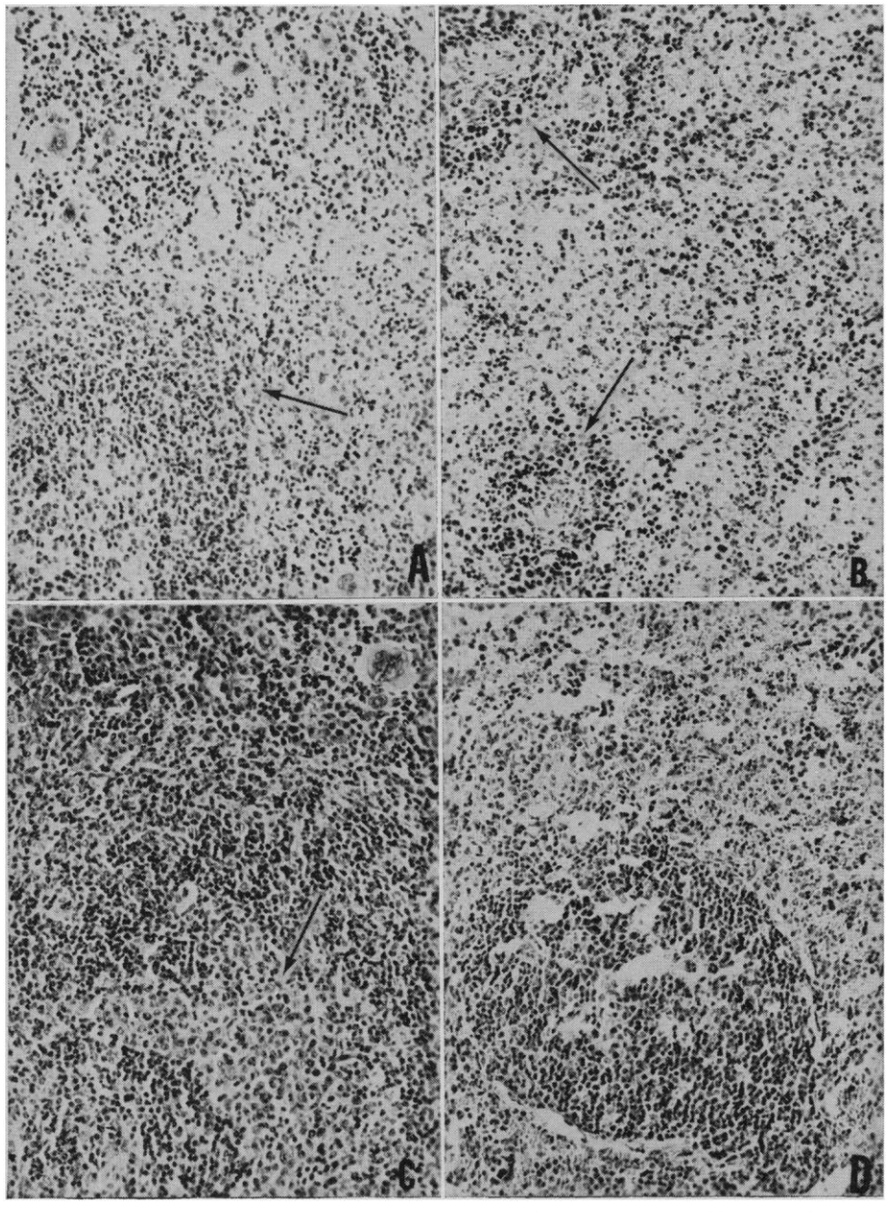

Fig. 2. Spleens from female Swiss mice after various treatments. Hematoxylin and eosin stain; (× 170). (A) Normal spleen, showing moderate activity in the germinal center, normal supply of small lymphocytes in the follicle (arrow), and moderate number of nucleated cells in the red pulp. (B) BPV-pretreated spleen, showing smaller than normal lymphoid follicles (arrows), decreased activity in the germinal center, depletion of small lymphocytes, and decreased number of nucleated cells in the red pulp around the follicle. (C) Spleen pretreated with phytohemagglutinin-M, showing much enlarged and coalesced lymphoid follicles, increased activity in the germinal centers (arrow), increased number of large pale cells, and large number of nucleated cells in the red pulp. (D) BPV followed by phytohemagglutinin-M; spleen shows lack of significant stimulation, that is, the lymphoid follicle is sharply outlined with decreased number of small lymphocytes, and there is a small number of nucleated cells in the widened red pulp around the follicle.

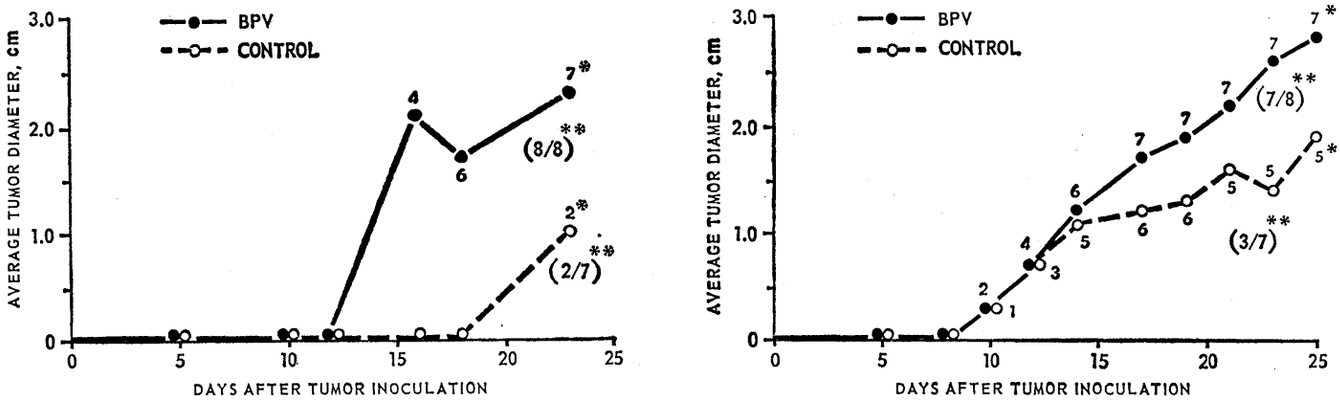

Fig. 1. Growth of a subcutaneous lymphoma transplant in normal mice and mice injected with BPV. Single asterisk indicates number of mice with tumor on each measurement. Double asterisks indicate total number of mice with tumor growth. (Left) BPV injected intravenously 4 days prior to tumor inoculation. (Right) BPV injected intravenously 7 days after tumor inoculation. Two mice in the control group rejected tumor transplants on further observations.

Thus, treatment of mice with BPV brought about a state of immunological deficiency, as suggested by the enhanced growth of transplanted tumors and, further, by the histological findings showing markedly impaired proliferative potential of lymphoid cells in the spleen. It has been shown by Morse that BPV induces a hyperlymphocytosis by massive mobilization of lymphocytes from all lymphoid organs; lymphocytopoiesis was not stimulated (*11*). In the experiment described here, all the lymphocytes mobilized may have been strongly committed to the complex antigens of BPV, and apparently there was no active lymphocyte formation. This unavailability of noncommitted lymphocytes at the time of the second antigenic challenge, that is, the tumor cell inoculum, may have resulted in the immunological deficiency state. Since BPV is known to increase humoral antibody production in some systems (*12*), one may consider immunological enhancement as another possible cause of augmented tumor growth. This possibility, however, appears less likely because of the impaired proliferation of pyroninophilic cells in the spleen of BPV-pretreated mice.

Phytohemagglutinin-M is known to cause blastic transformation of small lymphocytes in vivo (*13*) as well as in vitro (*14*). The absence of "blast" cell proliferation in the spleen of BPV-pretreated mice after injection of phytohemagglutinin-M may also be considered as further evidence of lymphocyte depletion in this organ. Proliferation of reticulum cells after FA injection is a well-established phenomenon (*15*) and FA has been widely utilized to augment humoral antibody production. It appears significant that this powerful stimulation also failed to elicit any noticeable cell proliferation in BPV-pretreated spleens.

Other experiments show that FA and phytohemagglutinin-M, when injected prior to tumor inoculation, also enhance the growth of the subcutaneous lymphoma. However, unlike BPV, FA clearly inhibited the tumor growth, when injected 1 week after the inoculation of lymphoma cells (*16*).

An enhancing effect of various lymphoreticular stimulants on the immunological function of animals has been well documented. But an immunologically adverse effect has also been noted. It was found that FA, a well-known stimulant of antibody production, induced a more rapid death and larger tumor formation in mice if given in large doses (*17*). Rauscher *et al.* reported an increased susceptibility to subliminal doses of Rous sarcoma virus in chicks pretreated with FA. On the other hand, if given after virus inoculation, the same dose of FA enhanced antibody production against Rous sarcoma virus (*18*). Elves reported a suppression of hemolysin production against chicken erythrocytes in rats pretreated with phytohemagglutinin-M. Such treatment after immunization, however, resulted in an enhanced antibody production (*19*). Spreafico and Lerner have also demonstrated the immunosuppressive effect of phytohemagglutinin (*20*). *Corynebacterium parvum*, another powerful reticuloendothelial stimulant, has been shown to abolish host resistance to a tumor transplant (*21*).

Thus, it may be reasonable to postulate that treatment of animals with potent lymphoreticular stimulants causes "pre-commitment" or "syphoning-off," as expressed by Elves, of immunologically competent cells (small lymphocytes, "blast" cells, reticulum cells), so that they are no longer available to react to a subsequent antigenic challenge. It is interesting in this regard that some infections of man, including pertussis, are known to augment susceptibility to other infections or activate latent infections.

M. HIRANO*, J. G. SINKOVICS
C. C. SHULLENBERGER
C. D. HOWE
Department of Medicine, University of Texas M. D. Anderson Hospital and Tumor Institute, Houston 77025

References and Notes

1. J. L. Gowans, D. D. McGregor, D. M. Cowen, C. E. Ford, *Nature* **196**, 651 (1962).
2. D. Metcalf, in *Radiation Biology*, Proceedings of the Second Australian Conference on Radiation Biology (Academic Press, New York, 1959), p. 26.
3. J. F. A. P. Miller, A. H. E. Marshal, R. G. White, *Advan. Immunol.* **2**, 111 (1962).
4. A. S. Gordon, *Ann. N.Y. Acad. Sci.* **59**, 907 (1955).
5. D. D. McGregor, J. L. Gowans, *Lancet* **1964-I**, 629 (1964).
6. C. Martinez, J. Kersey, B. B. Papermaster, R. A. Good, *Proc. Soc. Exp. Biol. Med.* **109**, 193 (1962); W. J. Dempsten, B. Lennox, J. W. Boag, *Brit. J. Exp. Pathol.* **31**, 670 (1950).
7. J. F. A. P. Miller, R. C. Ting, L. W. Law, *Proc. Soc. Exp. Biol. Med.* **116**, 323 (1964).
8. G. A. Grant, J. F. A. P. Miller, *Nature* **205**, 1124 (1965); Y. Nishizuka, I. Nakakuki, M. Usui, *ibid.*, p. 1236 (1965).
9. J. G. Sinkovics, B. A. Bertin, C. D. Howe, *Nat. Cancer Inst. Monograph 22*, p. 349 (1966).
10. J. G. Sinkovics, M. Hirano, A. Szakacs, C. C. Shullenberger, C. D. Howe, in *Proliferation and Spread of Neoplastic Cells*, Proceedings of the 21st Annual Symposium of Fundamental Cancer Research, The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, Texas, 1967 (Williams and Wilkins, Baltimore, Md., in press).
11. S. I. Morse, *J. Exp. Med.* **121**, 49 (1965).
12. J. Munoz, *J. Immunol.* **90**, 132 (1963).
13. C. N. Gamble, *Blood* **28**, 175 (1966).
14. J. H. Robbins, *Science* **146**, 1648 (1964).

15. R. D. Moore, M. E. Lamm, L. A. Lockman, M. D. Schoenberg, *Brit. J. Exp. Pathol.* **44**, 300 (1963).
16. M. Hirano, J. G. Sinkovics, C. C. Shullenberger, C. D. Howe, in preparation.
17. M. D. Schoenberg and R. D. Moore, *Cancer Res.* **20**, 1505 (1960).
18. F. J. Rauscher, M. A. Fink, J. P. Kvader, *J. Nat. Cancer Inst.* **30**, 654 (1963).
19. M. W. Elves, *Nature* **213**, 495 (1967).
20. F. Spreafico and E. M. Lerner II, *J. Immunol.* **98**, 407 (1967).
21. G. Cudkowicz, in *Proliferation and Spread of Neoplastic Cells*, Proceedings of the 21st Annual Symposium on Fundamental Cancer Research, The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, Texas, 1967 (Williams and Wilkins, Baltimore, in press).
22. This work was supported by PHS research grant CA-7923, award K3-CA-16,747, and institutional grant FR-05511. Dr. J. J. Butler, Department of Pathology, kindly revised the histological material.
* Present address: Mount Sinai Hospital, New York 10029. Address report requests to J. G. Sinkovics, University of Texas M. D. Anderson Hospital, Houston.

21 August 1967

Multiple Temperature-Sensitive Spots Innervated by Single Nerve Fibers

Abstract. *Electrophysiological recordings were made from single nerve fibers which were specifically responsive to temperature changes of the skin of monkeys. Previous reports indicated that the receptive area on the skin of such preparations was a single small spot less than 1 millimeter in diameter. However, we found that the activity in a single thermally sensitive fiber increased when any one of eight individual spots on the skin was cooled. In other preparations two to six spots, each less than 1 millimeter in diameter, appeared to be innervated by a single fiber. The neural activity resulting from the cooling of one or several of these spots summed, and we suggest that this summation may be the neural analog of areal summation of thermal stimuli reported in psychophysical measurements.*

Although knowledge of the structural characteristics of cutaneous temperature receptors is lacking, it is desirable to describe their functional characteristics. These characteristics can be described in psychophysical or behavioral terms, and electrophysiological data can be obtained from measurements of changes in the activity of the peripheral nerve associated with changes in skin temperature. Correlations of the psychophysical and electrophysiological measurements of the effects of thermal stimulation provide information relevant to the nature of peripheral coding of thermal stimuli and to the processing of this information by the nervous system.

One functional characteristic of temperature reception which has been described in psychophysical terms is areal summation of thermal stimuli (*1*). As the area of skin stimulated is increased (up to about 1500 cm^2) the intensity of thermal stimuli required to produce a threshold sensation decreases. On the basis of these and other data concerned with summation, Hergert, Granath, and Hardy (*2*) have suggested that areal summation results from a convergence of sensory nerve activity at two different sites. Summation over small areas (less than 4 or 5 cm^2) results from endings whose branches converge near the periphery, whereas summation over large areas (greater than 4 or 5 to less than 1500 cm^2) results from convergence of axons on synaptic pools within the central nervous system.

Electrophysiological investigations in primates of peripheral thermally sensitive fibers (*3*) have failed to provide any evidence of the first type of convergence, and, to our knowledge, the problem of thermal summation in the central nervous system has not been investigated. In preparations in which such single specific peripheral fibers have been isolated, increased activity was induced by the cooling of only a single spot (not more than 1 mm in diameter) on the skin. Innervation of several sensitive spots by a single fiber has not yet been reported, even for the tongue and face of the cat (*4*).

We believe that we have discovered the neurological counterpart of small-area thermal summation determined by psychophysical methods. The data reported here are from one of eight preparations of single fibers taken from the radial and saphenous nerves of rhesus (*Macaca mulatta*) and stumptail (*Macaca speciosus*) monkeys. In these preparations a single thermally sensitive fiber (*5*) innervated two to eight spots on the skin. These spots were distributed over areas of up to 1.7 cm^2, and each spot in the cluster was no more than 1 mm in diameter. Twenty preparations have been obtained in which a single fiber innervated only one spot on the skin, but the data are insufficient to estimate the relative proportion of

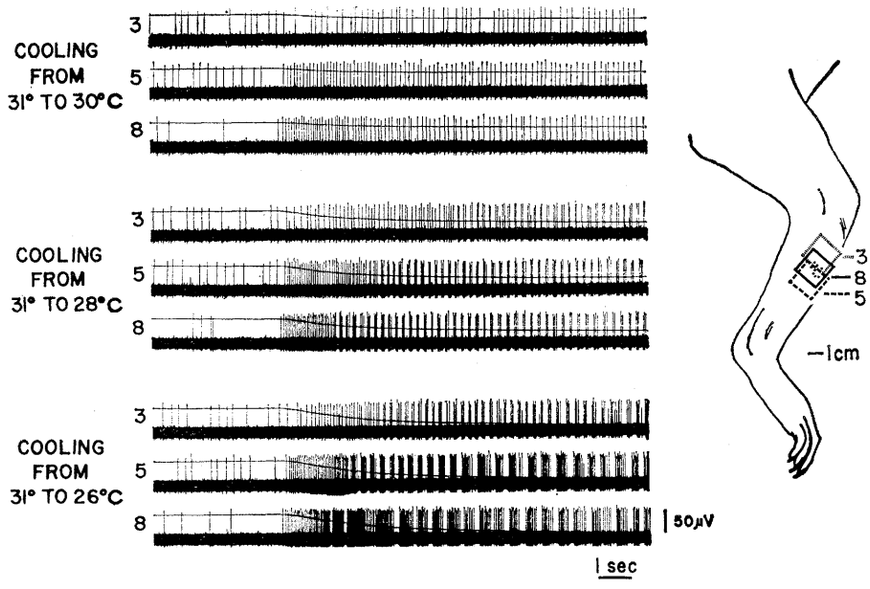

Fig. 1. The neural discharge of a single specific thermally sensitive fiber in response to cooling of the skin. Increased activity in the fiber was induced when any one of eight spots (shown to the right of the figure) was cooled with a small probe. Application of the probe to the skin between the spots failed to produce a response. Cooling first three, then five, and finally all eight of the spots by 1°, 3°, and 5°C with a large-area stimulator produced the neural activity shown here. The steady-state discharge, before the cool stimulus, was faster when three and five spots were stimulated than it was when all eight were stimulated. We attribute this to the fact that when the stimulator covered all eight spots, they are all maintained at 31°C. However, when only a portion of the spots were under the stimulator, the remaining ones were near skin temperature which, in this case, was 26°C. These few cooler spots were responsible for the higher steady-state frequency.