Species and Strain Dependence of the Response to a Granulocytosis-Promoting Factor (GPF) Extracted from a Mouse Tumor.* (31013)

L. DELMONTE AND R. A. LIEBELT (Introduced by R. Guillemin)
Department of Anatomy, Baylor University College of Medicine, Houston, Texas

Recently we reported a method for extraction and partial purification of a granulocytosis-promoting factor (GPF) from a transplantable mouse tumor, CE 1460(1). Leukocytosis-promoting activity, predominantly granulocytic in nature, has previously been demonstrated to be present in the plasma of leukapheresed rat(2) and human subjects(3), in the plasma of leukemic subjects(3), in plasma of non-hematologic-tumor bearing subjects(3), in a crude extract of a transplantable mouse tumor(4), and in extracts (leukopoietin-G) from normal bovine and porcine kidneys(5).

A discrepancy was noted between our data (1) and those of other investigators (2,3,5): the degree of granulocytosis induced by GPF in our intact (BALB/C $\times$ CE)F_1 hybrid mouse bioassay system far exceeded that reported for intact rats treated with "leukopoietin"-rich preparations by both Bierman *et al*(3,5) and Gordon *et al*(2).

Three alternate hypothetical explanations for this discrepancy could be proposed: 1) The active principle in the partially purified GPF is different from "leukopoietin." 2) The active principle in GPF and "leukopoietin" is the same but either (a) this principle is present in greater concentration in GPF or (b) its activity is partially masked by an inhibitor, the concentration of which is lower in GPF than in "leukopoietin" or (c) both. 3) Due to a species difference, granulocyte mobilization is evoked more readily in mice than in rats by the active principle in both GPF and "leukopoietin."

The purpose of the present study was to determine whether a species difference indeed exists in the granulocytic "response-potential" to GPF in mice and rats, and furthermore whether strain as well as species differences might possibly affect this "response-potential."

Materials and methods. GPF. The GPF used in this study was extracted from the CE 1460 mouse tumor by a modification of a previously reported method(6). Details of this modification will be reported elsewhere.

Animals. Two hundred and fourteen mice (15-25 g) of both sexes and of 4 inbred strains —CE†, BALB/C†, (BALB/C $\times$ CE)F_1 hybrid† and A_f†—and 85 male rats (130-150 g) of 2 random strains—Long-Evans‡ and Sprague-Dawley§—were used. The animals were housed in groups of 6 mice or 3 rats per cage and allowed Purina Lab Chow and water freely.

Bioassay method. Each animal received a single dose of GPF dissolved in 0.25 ml of pyrogen-free isotonic saline and injected into the tail vein. Doses of GPF ranged from 0.005 mg to 350 mg/kg of body weight. Pyrogen-free isotonic saline was used as the control. All bioassay injections were given between 9:00 and 10:00 A.M., in order to minimize individual variations which might be anticipated due to the normal diurnal leukocyte rhythm superimposed on the acute leukocyte release phenomenon evoked by the assay material.

Bioassays consisted of sampling venous tail blood immediately prior to and at 1,3,4,5,6,7,8 and 24 hours following a single injection of GPF solution or saline. Samples of blood (13 μl) were diluted with 1.3 ml of 1% ammonium oxalate in Unopette® disposable blood diluting pipettes, and total leukocyte numbers determined in a Spencer hemocytometer. Wright-Giemsa-stained blood smears were prepared for differential leukocyte counts.

Repeated bioassays were performed in in-

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† Kirschbaum Memorial Laboratory, Baylor Univ. College of Medicine, Houston, Texas.

‡ K-G Farms, Inc., Parsippany, N. J.

§ Charles River Breeding Laboratories, Inc., Wilmington, Mass.

dividual male CE mice given 2 injections and in individual Long-Evans rats given 4 injections of GPF (i.v.) at 3- to 4-day intervals.

The *peak leukocyte response level* was defined(1) as the highest degree of leukocytosis manifested by a given animal during the 8-hour period following a single injection of GPF. The *minimal effective dose* (MED) of GPF for a given mouse or rat strain was defined as the dose level at which the leukocyte response differed significantly ($P<0.05$) from the "non-specific" response evoked in the respective strain by the injection of pyrogen-free isotonic saline. The *minimal optimal dose* (MOD) was defined as the smallest dose inducing the maximal granulocyte response to GPF of which a given mouse or rat strain is capable.

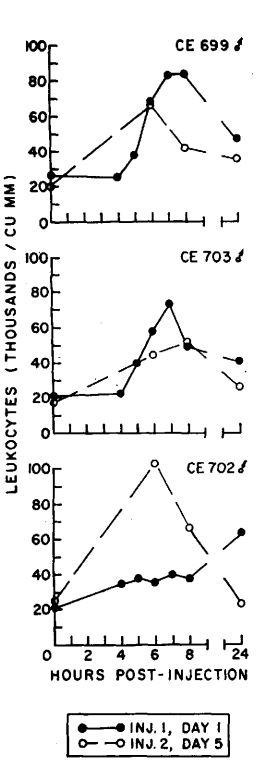
Results. In all mouse and rat strains studied, the response to both GPF and isotonic saline was characterized by a progressive albeit transitory leukocytosis with peak levels occurring 3-7 hours following the injection, and by a gradual return of leukocytes to pre-injection levels by 9 or more hours. The individual lines in Fig. 1 and 2 exemplify the characteristics of the GPF-induced leukocytosis in CE mice and Long-Evans rats. During the period of 3-8 hours following injection, granulocytes comprised 95-98% and 50-70% of the circulating leukocytes in GPF-treated and isotonic saline-treated animals, respectively, as compared to 15-30% in untreated controls of both species. By 5-7 hours following administration of GPF, approximately 5% of the circulating granulocytic forms consisted of stab cells and metamyelocytes.

In mice, the MED of GPF per kg of body weight was 0.005 mg for the CE, BALB/C and (BALB/C $\times$ CE)F_1 hybrid strains and 5.0 mg for the A_f strain. The MOD of GPF per kg of body weight was 5 mg for the first 3 and 50 mg for the latter strain, respectively. At optimal dose levels of GPF, the peak leukocyte response averaged 60,000-80,000/cu mm for CE, 50,000-70,000/cu mm for BALB/C and 65,000-85,000/cu mm for (BALB/C$\times$CE)F_1 hybrid mice, and exceeded 100,000/cu mm in 20% of CE and (BALB/C $\times$ CE)F_1 hybrid as well as in 10% of BALB/C mice (Fig. 3). By contrast, in A_f mice given optimal dose levels of GPF, the peak leukocyte response averaged only 20,000-30,000/cu mm, exceeded 40,000/cu mm in 20% and never exceeded 50,000/cu mm (Fig. 3). This strain difference was mirrored both in the normal leukocyte range—8,000-20,000/cu mm for the 3 high-response strains and 6,000-16,000/cu mm for the low-response strain—and in the peak leukocyte response to isotonic saline—20,000-45,000/cu mm and 9,000-20,000/cu mm for the high- and low-response strains, respectively (Fig. 3).

In rats, the MED of GPF per kg of body weight was 0.0007 mg for the Long-Evans and 0.007 for the Sprague-Dawley strain. The MOD of GPF per kg of body weight was 7 mg for Long-Evans and 70 mg for Sprague-Dawley rats. At optimal dose levels, the peak leukocyte response averaged 60,000-80,000/cu mm and ranged beyond 100,000/cu mm in 20% of the Long-Evans rats (Fig. 4). In Sprague-Dawley rats, on the other hand, the peak leukocyte response averaged only 25,000-35,000/cu mm, ranged beyond 45,000/cu mm in less than 10% and never exceeded 50,000/cu mm (Fig. 4). Again the strain difference in responsiveness was reproduced both in the normal leukocyte range—9,000-20,000/cu mm and 5,000-15,000/cu mm, respectively—and in the peak leukocyte response to isotonic saline—20,000-50,000/cu mm and 10,000-19,000/cu mm, respectively—for the Long-Evans and Sprague-Dawley strains (Fig. 4).

In both mice and rats, it was possible to induce transitory leukocytosis but not to maintain sustained leukocytosis with multiple injections of GPF. As can be seen from the examples of response of CE mice to 2 doses of GPF given at 4-day intervals (Fig. 1) and of Long-Evans rats given 4 doses of GPF at 3- to 4-day intervals (Fig. 2), the highest peak granulocyte response in a given animal occurred variably after the first (Fig. 1), second (Fig. 1 and 2), or even the third of the consecutive injections of GPF (Fig. 2). The usual pattern for multiple injections, however, was for a gradual decrease in peak granulocyte response levels with the third and fourth dose of GPF (Fig. 2).

Discussion. It is well known that the normal mammalian blood picture shows species and strain differences in hematologic parameters including the cytology, cytochemistry and



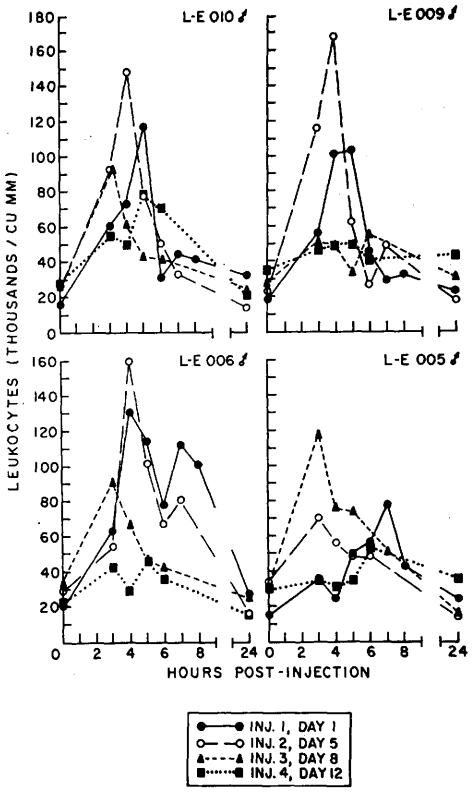


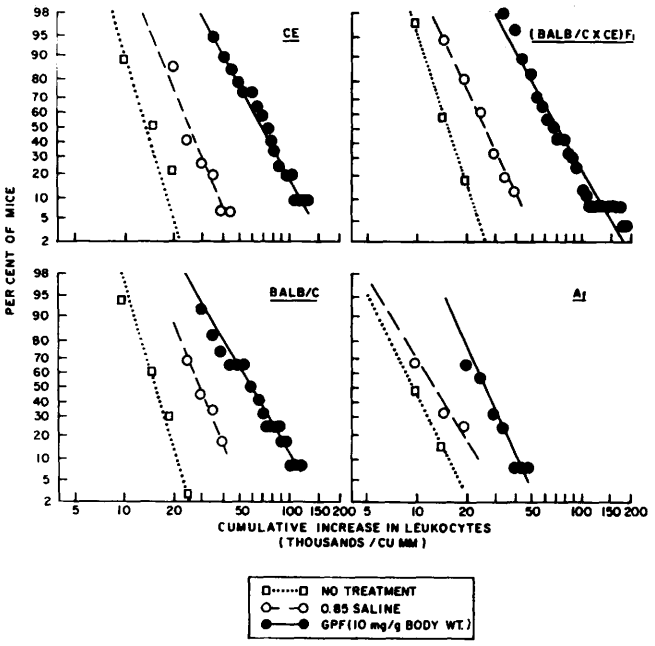
FIG. 1 (left). Repeated transitory leukocytosis in adult male CE mice receiving 2 i.v. injections of 15 mg GPF/kg body weight, at 4-day intervals.

FIG. 2 (right). Repeated transitory leukocytosis in adult male Long-Evans (L-E) rats receiving 4 i.v. injections of 15 mg GPF/kg body weight, at 3- to 4-day intervals.

number of the formed elements as well as the kinetics of hemopoiesis. Therefore, it is only to be expected that species and strain differences should also be evident in the potential for rapid granulocyte mobilization under the influence of a granulocytosis-promoting factor such as GPF.

The present studies reveal the existence of marked strain differences in the ability of two rodent species, mice and rats, to respond to GPF. These differences are apparently dependent on two factors: 1) the amount of readily available active principle required to institute a significant ($P < 0.05$) granulocytic response and 2) the potential of the host's granulopoietic tissue and granulocyte reserves to respond to the stimulus of this active principle. Our data indicated that both these factors may be reflected by the parameters of 1) the normal peripheral leukocyte range and 2) the response to pyrogen-free isotonic saline in a given mouse or rat strain, so that from these two parameters the probable strain-specific response to GPF can apparently be predicted.

The characteristics of the granulocyte release curve in GPF-treated mice indicate a rapid mobilization of granulocytes from the marginated and extravascular reserve pool, granulocytosis rising to a peak and then gradually decreasing as the reserve compartment is depleted. Furthermore, the observation of immature granulocytic forms in the circulation within 5-7 hours after injection of GPF, coupled with unpublished observations from our laboratories showing an increase in colony-forming units in the bone marrow of intact and irradiated GPF-treated mice(6), suggests that GPF also acts at the level of the bone marrow. Similar stimulation of the bone marrow compartment has been demonstrated by Gordon *et al*(2,7) who perfused isolated rat hind limbs with "leukopoietin"-rich serum, and by Bierman(8) who presented preliminary evidence



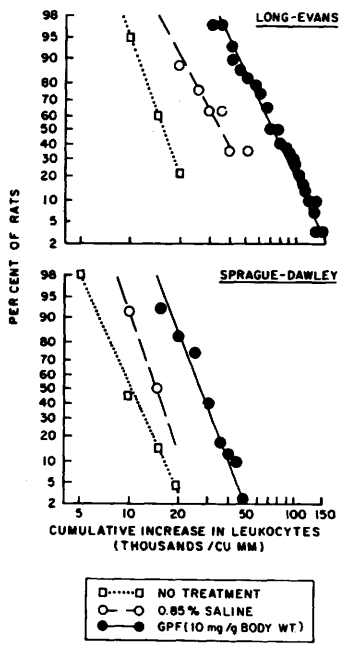


FIG. 3 (left). Strain difference in peak granulocyte response obtained in mice with a single i.v. injection of optimal doses of GPF. (Probability —log response.)

FIG. 4 (right). Strain difference in peak granulocyte response obtained in rats with a single i.v. injection of optimal doses of GPF. (Probability —log response.)

that leukopoietin-G increased the number of colony-forming units in mouse bone marrow.

Preliminary unpublished data from our laboratories showed that when GPF was administered at 8-, 12- or 24-hour intervals over a 21-day period, the first 2 injections evoked transitory but not sustained granulocytosis, after which no further peripheral granulocyte response could be evoked. This suggested either an exhaustion of the granulocyte reserves or an accumulation of high titers of a hypothetical (exogenous or endogenous) granulocyte-release inhibitor or development of pharmacologic resistance. In our present studies, the spacing of consecutive injections of GPF at 3- to 4-day intervals apparently permitted only partial recovery of the granulocyte reserve compartment, since there usually was a progressive decrease in the level of granulocyte response to the third and fourth consecutive dose of GPF. To date, neither the accumulation of a hypothetical inhibitor of granulocytosis nor the gradual development of pharmacologic resistance to GPF have been ruled out as factors in the above mentioned phenomenon.

Unpublished data from our laboratories have shown that GPF can be extracted not only from the CE 1460 mouse tumor but also from normal mammalian kidney tissue(6), the same source as that used by Bierman(5) for leukopoietin-G. It is therefore considered probable that GPF contains the same active principle as "leukopoietin." Only a direct bioassay comparison in a given mouse or rat strain could determine whether the greater granulocyte release promoted with GPF, as compared to the response reported for intact rats given plasma "leukopoietin"(2,3) and bovine and porcine and kidney leukopoietin-G (5) is due 1) to a strain difference in the bioassay systems or 2) to a greater concentration of the active (granulocytosis-promoting) principle and/or a lower concentration of a hypothetical granulocyte-release inhibitor substance in the partially purified GPF prepared by our method(1) or 3) to a combination of these factors.

Summary. Marked strain differences were observed in the degree of granulocytosis which could be induced in both mice and rats by a partially purified granulocytosis - promoting

factor (GPF) extracted from a transplantable mouse tumor, CE 1460. The strain differences appeared to be reflected in both the normal leukocyte range and the response to isotonic saline, parameters from which the strain-specific response to GPF could apparently be predicted. It was postulated that the active principle in GPF may be the same as that present in "leukopoietin" preparations from plasma and tissue sources. Furthermore, it was hypothesized that the greater granulocyte release promoted by GPF, as compared to that promoted by "leukopoietin"-rich preparations, might conceivably be due 1) to species or strain differences in the bioassay systems or 2) to a greater concentration of the active (granulocytosis-promoting) principle and/or a lower concentration of a theoretical granulocyte-release inhibiting substance in the partially purified GPF prepared by our method or 3) to a combination of these factors. These results indicate that genetic (strain and species) differences should be taken into consideration in evaluating the biologic activity of GPF and possibly also of "leukopoietin."

1. Delmonte, L., Liebelt, R. A., Science, 1965, v148, 521.

2. Gordon, A. S., Neri, R. O., Siegel, C. D., Dornfest, B. S., Handler, E. S., Lo Bue, J., Eisler, M., Acta Haematol., 1960, v23, 323.

3. Bierman, H. R., Marshall, G. J., Maekawa, T., Kelly, K. H., Plante, P., J. Clin. Invest., 1958, v37, 877.

4. Lappat, E. J., Cawein, M., Cancer Research, 1964, v24, 302.

5. Bierman, H. R., Ann. N. Y. Acad. Sci., 1964, v113, 753.

6. Reported at meeting of Southwestern Section, Am. Soc. for Cancer Research, Galveston, Texas, Oct. 29-30, 1965.

7. Gordon, A. S., Siegel, C. D., Dornfest, B. S., Handler, E. S., Lo Bue, J., Trans. N. Y. Acad. Sci., 1960, v23, 39.

8. Bierman, H. R., Exp. Hematol., 1964, no. 7, 83.

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Effects of Various Soybean Products on Flatulence in the Adult Man.* (31014)

F. R. STEGGERDA, E. A. RICHARDS AND J. J. RACKIS

Department of Physiology and Biophysics, University of Illinois, Urbana, and Northern Regional Research Laboratory,† Peoria, Ill.

The potential of soybeans as a source of vegetable protein for human consumption has long been recognized(1-3). Although remarkable advances have been made in developing the uses of soybeans, there still remains a flatus-producing factor which must be identified and eventually removed so that the soybean food products available will become acceptable for human consumption. In our investigation, various soybean products were tested for their capacity to produce flatulence, the results of which are reported here.

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† A Laboratory of the Northern Utilization Research and Development Division, Agri. Research Service, U. S. Dept. of Agriculture.

Materials and methods. Four adult males served as subjects for the experiments. Each experiment lasted 6 days during which time the subject consumed at each meal a measured amount of muffins and mush containing known amounts of wheat flour, soybean meal, and corn meal. This diet was supplemented with specific amounts of hamburger, skim milk, and fruits in the form of orange juice, bananas, and canned pears. Noncaloric syrup was used on the muffins and soy-corn mush at each meal. Adequate vitamin supplements were also taken each day. The caloric content of the diet was 2334 calories per day, and the total amount of soybean products (Fig. 1) consumed each day was 146 g. For purposes of comparison, soybean meal was replaced in some experiments with ground navy bean meal. Previous to use, the soy-