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QUANTITATIVE STUDIES OF HUMAN LEUKOCYTIC AND FEBRILE RESPONSE TO SINGLE AND REPEATED DOSES OF PURIFIED BACTERIAL ENDOTOXIN *

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Bacterial endotoxins injected intravenously in small doses into animals and man produce, among other effects, a transient granulocytopenia followed by a marked granulocytosis (1–7). Several comprehensive reviews have recently appeared which deal with the wide spectrum of biologic effects of these substances, including their relationship to the pathogenesis of fever, the development of tolerance to repeated administration, and effects upon host resistance to bacterial infection (5, 8, 9).

Recent studies utilizing radioisotopic cell-labeling techniques have increased considerably the understanding of granulocyte kinetics. Athens and his associates estimate the total blood granulocytes in man to be made up of nearly equal compartments of circulating cells and cells marginated and sequestrated within the capillary bed (10). The granulocytic elements in the marrow which are no longer capable of proliferation consist of a reserve of mature cells and cells in various stages of maturation which has been estimated to number 20 to 70 times the total blood granulocytes (7, 11, 12). Craddock, Perry and Lawrence consider this marrow granulocyte reserve to be the source of the granulocytes that enter the circulation in response to leukocytopheresis (11) and endotoxin (1). Athens and co-workers have added evidence that the source of the endotoxin granulocytosis is the bone marrow (10). No appreciable return of granulocytes to the circulation from extravascular spaces has been demonstrated. Craddock and others have suggested that the magnitude of the increase in circulating granulocytes after endotoxin administration may relate directly to the size of the marrow granulocyte reserve and thereby serve as a means of assessing marrow function (7, 12).

Because of their potency, the clinical dosage of purified endotoxins has been confined to a relatively narrow range. Recent investigators, using Pyrexal, the endotoxic polysaccharide derived from *Salmonella abortus equi,* have been able to obtain a granulocytic effect at a dose that did not produce severe systemic reactions (7, 13). There is some disparity in dose levels at which unpleasant side effects were observed, but it is noteworthy that Keller and Heilmeyer reported no essential difference in the size of the granulocyte response where side effects were rare, as compared with doses of 0.1 to 0.2 μg where there was a fairly high incidence of headache, chills and fever (13).

The broad objective of the present study was to determine the usefulness of endotoxin stimulation of granulocytosis as a measure of bone-marrow function. The approaches to this included: *1*) a study of dose-response relationships for granulocyte increase, fever and selected other endotoxin effects; *2*) determination of the variability of response among a group of normal volunteers; and *3*) a study of the effect of repeated daily doses of endotoxin on the various parameters of response.

MATERIALS AND METHODS

The subjects studied were prisoners at the U. S. Federal Penitentiary, Lewisburg, Pa. Men between the ages of 21 and 45, free of intercurrent infection and having no history or physical findings suggestive of hematologic and cardiovascular disease, or pathology involving the liver or spleen, were chosen at random from a group who volunteered to participate in the project. Subjects with a baseline white blood cell count over 10,000 cells per mm^3 or baseline temperature in excess of 38° C on the morning of the test were not included. In the graduated-dose study, eligible subjects were allotted to a given treatment in the order in which their names

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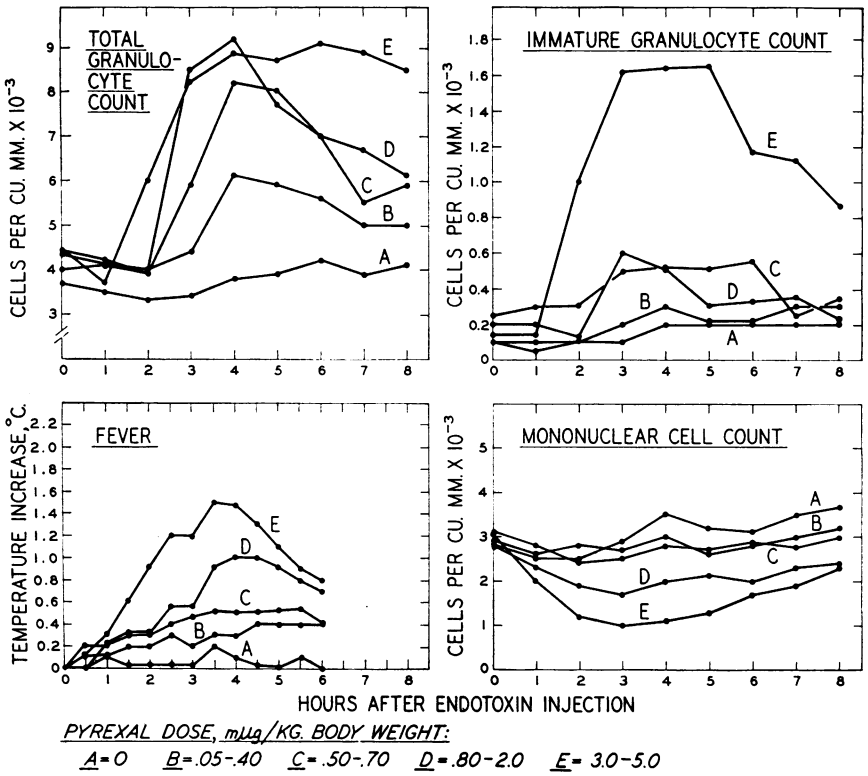

FIG. 1. DEVELOPMENT OF FEVER, TOTAL AND IMMATURE GRANULOCYTOSIS AND MONONUCLEOCYTOPENIA AFTER INJECTION OF GRADED DOSES OF ENDOTOXIN. COMPOSITE OF THE FOLLOWING NUMBER OF SUBJECTS FOR EACH DOSE RANGE: 7(A), 22(B), 6(C), 16(D) AND 18(E).

appeared on the list. In selection of subjects for the repeated-dose studies we attempted to obtain men of apparent emotional stability who would not be likely to drop out during the course of the test. The men were admitted to the prison hospital the night preceding the test and remained in bed during the test period, except for mealtime and bathroom privileges. The men participating in the repeated-dose study lived in the hospital during the entire time they were being tested.

Rectal temperatures were taken with a clinical, centigrade thermometer just before injection and every half-hour thereafter for 8 hours. Thermometers were left in place for a minimum of 3 minutes. Total white blood counts were made in the Coulter electronic particle counter by the method of Richar and Breakell on blood obtained by finger puncture (14). Differential counts of 100 to 300 cells on simultaneously obtained blood smears stained with Wright's stain were performed by experienced technician inmates under the supervision of one of us (R.M. or E.F.). Counts were done at zero time and then hourly for 8 hours after the injection. Pyrexal (lot 010), in ampules of 2 ml = 1 μg, was obtained from Wander SA, Berne, Switzerland. This product was selected for the study because it has been purified and standardized, and substantial clinical experience has been accumulated in its use (2, 7, 13). Immediately before the injection 10- or 100-fold dilutions were made in pyrogen-free vials containing either distilled water or physiologic saline solution. New, pyrogen-free disposable needles and plastic syringes were employed for dilution and injection. Control subjects receiving either saline or water alone were included, with otherwise identical technique, on three separate days. The identity of the control group was not known to either the laboratory or ward assistants. Endotoxin dosage ranged from 0.05 to 5 mμg per kg body weight.

The temperature curves were plotted on standard graph paper with 1 hour and 1° C equaling 1 inch (2.54 cm). The curves were terminated at 6 hours to minimize the artifact otherwise introduced by diurnal temperature variations (15). At that time the febrile responses were well past their peaks. The fever index was determined by measuring the area in centimeters squared under the curve with a compensating polar planimeter (Keuffel and Esser, 4236 M). This index correlated better with endotoxin dosage than did peak temperature rise and other measurements (15, 16).

RESULTS

Graduated-dose study. An upper dosage limit of 5 mμg per kg was not exceeded because of the

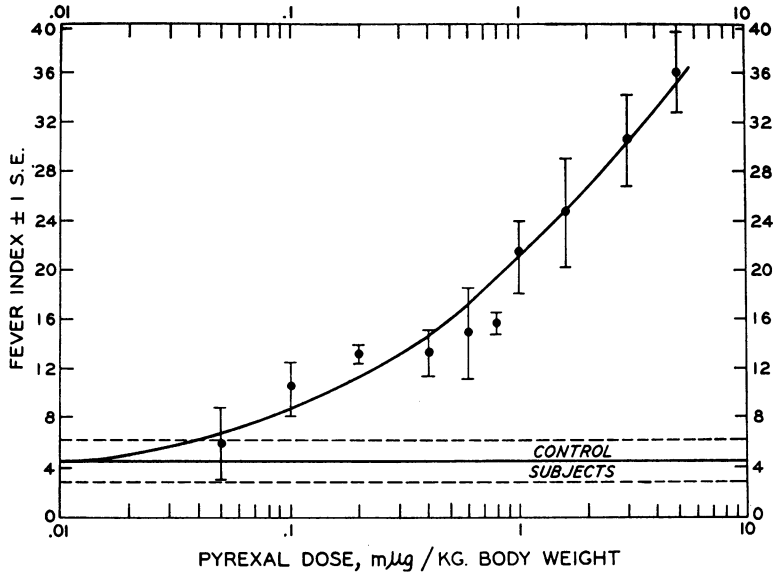

FIG. 2. RELATIONSHIP OF DOSE OF ENDOTOXIN TO MEAN FEVER INDEX.

frequency and severity of toxic symptoms at that level. These included, in descending order of frequency, mild to marked rigor 60 to 100 minutes after injection, feverish sensations, headache and generalized muscular aching, nausea and malaise. Chilling, headache and nausea were fairly severe in 3 of the 12 subjects receiving 5 mμg per kg. With doses of 0.5 mμg per kg or less, such symptoms were rare, and did not occur in most cases until the 1.0 mμg per kg level was reached. Significant hypotension was not encountered in these healthy subjects.

The time course of the development of fever with increasing doses of Pyrexal is shown in Figure 1 and the dose-response plot for fever index in Figure 2. The seven control subjects had a mean fever index of 4.7 with a standard deviation of 3.7. An index greater than 12 was considered to be a significant response.[1] The percentage of subjects showing indices in excess of 12 at the various dose levels is shown in Table I. The highest temperature, 39.8°, was recorded 3 to 4 hours after injection of 5.0 mμg per kg. A biphasic temperature response, characteristic of higher doses of pyrogen in animal experiments, was not seen in this study (8). The dose-response plot for fever (Figure 2) shows an essentially linear relationship from 0.6 mμg per kg upward, and as a substantial majority of subjects had significant fever response above this level, this value was taken to represent the minimal pyrogenic dose.

TABLE I

Percentage of subjects reacting to an intravenous injection of Pyrexal with elevated total and immature granulocyte counts and fever

Dose range	Subjects	Subjects with positive reaction: Granulocytes (>2,200)	Immature granulo. (>500)	Fever (F.I.*>12)
mμg/kg	no.	%	%	%
0	7	0	0	0
0.05–0.4	22	54	14	41
0.5 –0.7	6	83	67	67
0.8 –1.6	16	100	56	94
3.0 –5.0	18	100	100	100

* Fever index.

The granulocyte count of the seven control subjects during the 8-hour observation period showed a mean positive deviation of 860 with a SD of 670 cells per mm³. Taking an increment of 2,200 cells per mm³ as a significant reaction,[1] the percentage of subjects that reacted as the dose level was increased is shown in Table I. A single subject reacted to a dose of 0.05 mμg per kg, significant mobilization was seen consistently at 0.50 mμg per kg or more, and no significant change in the increment occurred at doses above 0.8 mμg per kg (Table II). For the 34 subjects given the

[1] Mean ± 2 SD. An untreated subject would have approximately 1 chance in 40 of showing a value higher than this.

endotoxin in amounts ranging from 0.8 to 5.0 mμg per kg, the mean granulocyte increment was 5,615 with a standard error of only 246 cells per mm³ (SD 1,430 cells per mm³). The mean ratio of mobilized to baseline counts in this group was 2.45 (SE 0.10).

The dose-granulocyte response is presented graphically in Figure 3. The horizontal heavy line represents the mean baseline granulocyte count for the 69 subjects and was 4,450 (SE 60) cells per mm³. The response increased with dose up to 0.8 mμg per kg, after which no further increase in granulocyte increment was observed.

The time of peak granulocyte count after endotoxin injection varied with dose as shown in Figure 1. At the lowest doses there was a slow ascent to a peak in 5 to 6 hours, while doses of 0.8 to 1.6 mμg per kg caused a fairly sharp peak at 3 to 4 hours. Higher doses produced a more prolonged response, with the elevation persisting at the end of the 8-hour observation period.

Nonsegmented granulocytes were recorded as immature forms, care being taken to insure uniform criteria in the counts. Metamyelocytes were found infrequently and were included in this category. The mean maximal increment in immature forms for the seven control subjects was 236 (SD 131) cells per mm³. An increase of more than 500 cells per mm³ was therefore considered significant.[1] The percentage of subjects showing significant response at increasing dose levels is given in Table I and a dose-response plot in Figure 4. Table II shows the mean peak increments in these cells for the dose levels that gave maximal values for total granulocyte count. It may be noted that no marked increase in young forms occurred until a dose level was reached that was more than twice that required for the maximal total granulocyte response.

TABLE II

Mean increment in total and immature granulocyte peak count and fever index in subjects given an intravenous injection of Pyrexal sufficent to produce a maximal peak granulocytic response

Dose	Subjects	Mean increment ±SE		Mean F. I. ±SE
		Total	Immature	
mμg/kg	*no.*			
0.8	4	5,425±1,120	725±218	16.5±0.9
1.0	7	5,414± 557	657±136	22.7±3.6
1.6	5	5,300± 765	770±265	26.0±4.7
3.0	6	6,167± 615	1,550±223	32.2±3.9
5.0	12	5,650± 330	2,096±235	38.1±3.6

A mononucleocytopenia occurred during the first few hours after injection of the higher doses of Pyrexal, as shown in Figure 1. In the seven controls there was a mean maximal decrease of

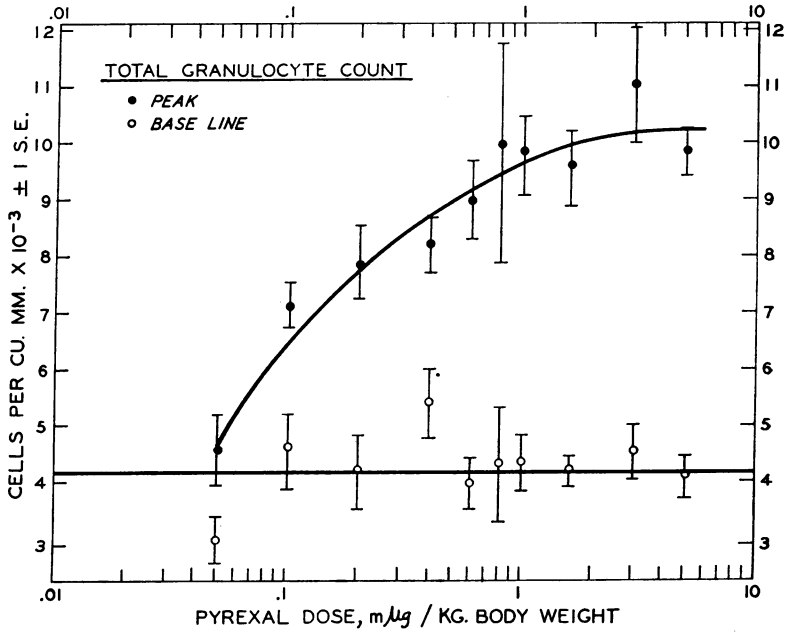

FIG. 3. RELATIONSHIP OF ENDOTOXIN DOSE TO MEAN GRANULOCYTE COUNT.

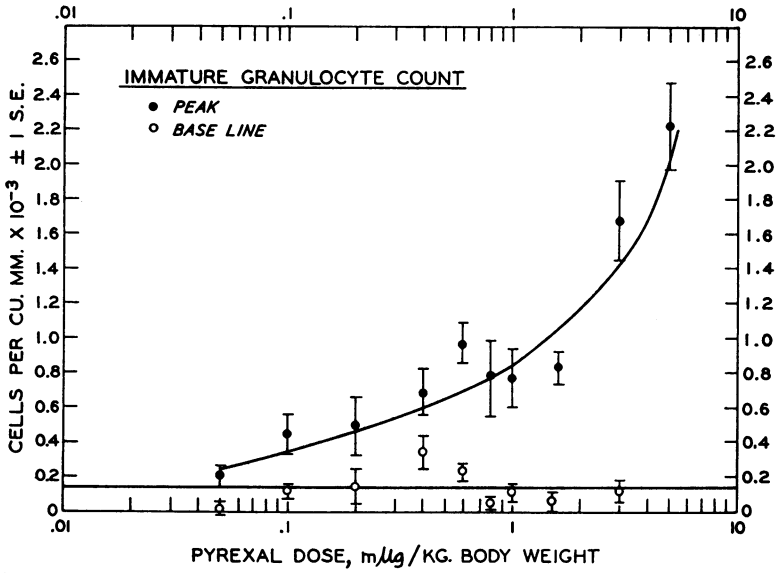

FIG. 4. RELATIONSHIP OF ENDOTOXIN DOSE TO MEAN IMMATURE GRANULOCYTE COUNT.

300 cells per mm³ (SD 900) from an initial mean count of 2,909 (SE 95) mononuclear cells per mm³. At doses of 0.8 to 2.0 mμg per kg there was a mean maximal drop of 1,438 (SE 261) cells per mm³, and at doses of 3.0 to 5.0 mμg the decrease amounted to 2,228 (SE 211) cells per mm³ at an average of 3 hours after injection.

Repeated-dose study. Changes in fever index,

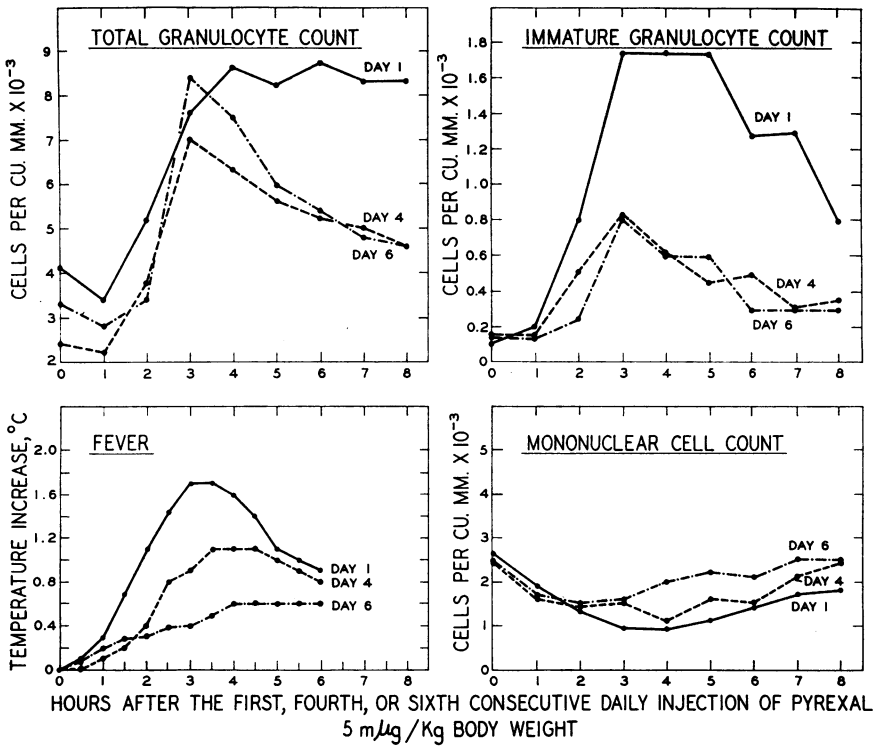

FIG. 5. EFFECT OF REPEATED INJECTIONS OF ENDOTOXIN ON THE DEVELOPMENT OF FEVER, TOTAL AND IMMATURE GRANULOCYTOSIS AND MONONUCLEOCYTOPENIA; COMPOSITE OF NINE SUBJECTS.

subjective effects and leukocyte counts were studied in nine subjects given repeated doses of 5.0 mμg per kg. All subjects received this dose for 4 days. Three of these men were continued on the regimen for a total of six and three for a total of eight consecutive daily injections.

The severity of unpleasant side effects declined progressively; after the third day complaints were infrequent, being virtually absent by Day 6. Headache was the single complaint persisting longest among these men. Figure 5 shows the development of fever, granulocytosis and lymphopenia on Days 1, 4 and 6. Mean fever indices on successive days are shown in Figure 6. From an initial fever index of 42 on Day 1 there was a progressive decline to a mean index of 12 for the three subjects continued for 8 days. This is the value previously estimated to be the upper limit of the control response. Two additional subjects given 1.0 mμg per kg for 7 consecutive days showed indices of 11 and 20, respectively, on Day 1, and 11 and 15 on Day 7. On Day 8 each was given an injection of 5.0 mμg per kg and the indices remained essentially unchanged (11 and 11).

Changes in granulocyte baseline and peak levels are shown in Figure 7 for the nine subjects given four consecutive daily injections of 5.0 mμg of the endotoxin per kg. The initial mean baseline, 4,480 (SE 356) per mm³, is set at zero in the upper panel of the figure. Consecutive preinjection counts were progressively lower, reaching a mean of 2,414 on Day 4. Mean preinjection counts thereafter, on the subjects continued for 6 and 8 days, varied irregularly from 2,650 to 3,650 cells per mm³. The mean peak count after each injection also declined on successive days, while the increments above each day's baseline remained remarkably constant. The mean daily increment for the entire period of 8 days averaged 4,859 (SE 356) cells per mm³. Two volunteers given 1.0 mμg per kg for 7 consecutive days followed the same general pattern, their mean daily increment averaging 4,693 (SE 276) cells per mm³. On Day 8, when challenged with 5.0 mμg per kg, their peak increments were 7,700 and 4,500, respectively.

As in the graduated-dose study, immature granulocytes departed considerably from this behavior. Baseline counts in the subjects given 5 mμg per kg per day were initially quite low and did not vary appreciably. The peak counts for Days 1 and 2 were, respectively, 2,350 and 2,200 (SE 275) cells per mm³, but dropped sharply on Days 3 and 4 (Figure 7). For the remainder of the test the counts were irregular, but the mean peak daily increment remained essentially the same, being 1,121 (SE 118) cells per mm³. The two men given seven daily injections of 1 mμg showed a mean peak elevation of 600 immature cells per mm³. On Day 8, after the challenge with 5 mμg, the average increment of immature cells in these men was only 725 cells per mm³.

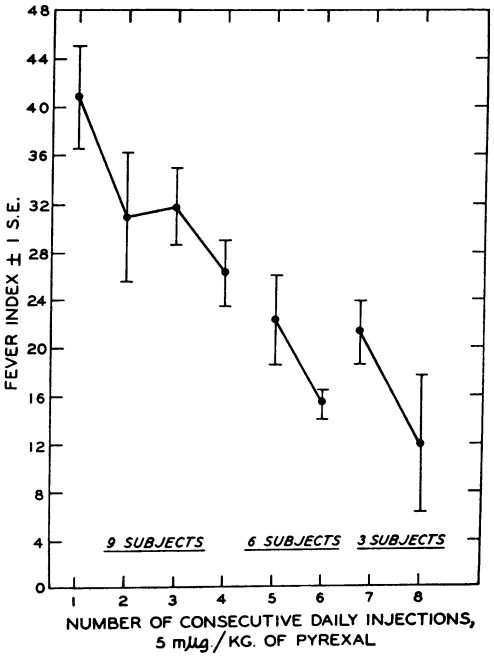

FIG. 6. DEVELOPMENT OF TOLERANCE TO ENDOTOXIN AS DETERMINED BY FEBRILE RESPONSE.

Three of the subjects who had received 5 mμg per kg per day for 4 consecutive days were given three additional such injections after a 2-day rest. The fever index continued its downward trend to a mean value of 11 after the last injection. The peak granulocyte increment remained high, however, averaging 5,000 after the final injection. Thus, the development of tolerance to the pyrogenic property of the endotoxin was not accompanied by tolerance to the property responsible for the granulocytic response.

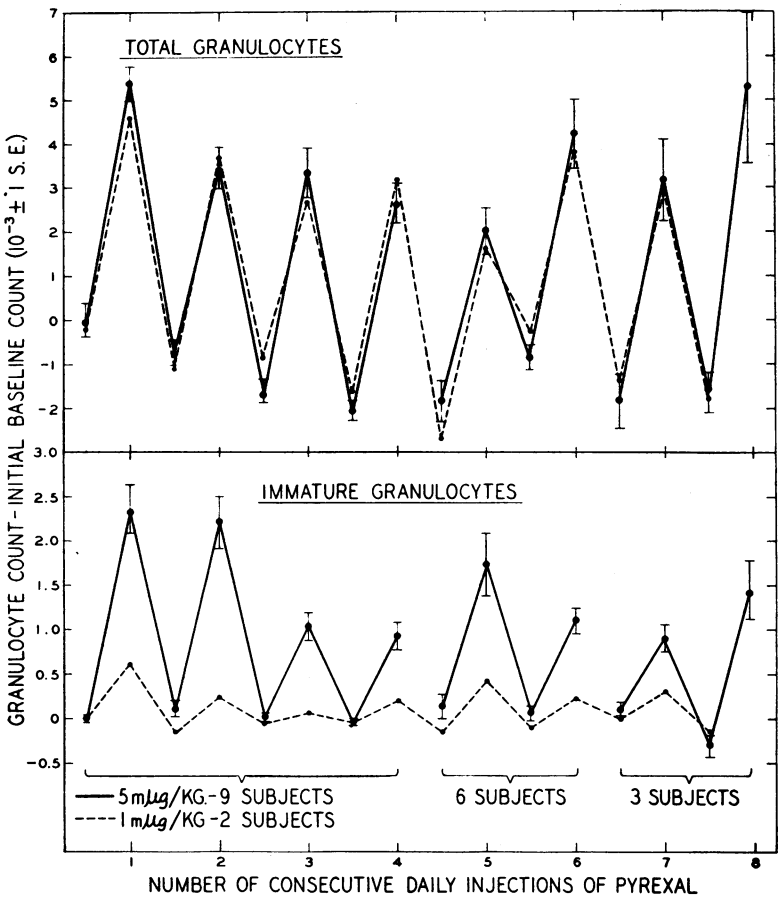

FIG. 7. EFFECT OF REPEATED INJECTIONS OF ENDOTOXIN ON GRANULOCYTE INCREMENTS AND BASELINE VALUES.

DISCUSSION

In this study we sought to achieve an understanding of the patterns of normal human response to the intravenous injection of small doses of purified bacterial endotoxin, sufficient to support its application to the testing of granulocyte reserves. It was particularly important to determine whether the various parameters of reaction to endotoxin increase concurrently with increasing dose and whether they disappear simultaneously as tolerance is developed, or whether a dose range and a stage in the onset of tolerance would be found in which the expression of one or another of the reactions predominates.

Although the degree of individual variation observed in various parameters of response did not appear to be excessive, certain differences suggest that specific factors influenced the results. For example, there were four volunteers of Puerto Rican birth who showed fever indices significantly below the means for their respective dosage groups ($p < 0.05$). Patients with previous malarial infection may have a subnormal response to the intravenous injection of typhoid vaccine (17), presumably because of a proliferative effect on reticuloendothelial elements. A history of previous plasmodial or other parasitic infection could not, however, be elicited from these men. Physical expression of the psychological reaction to the experimental situation as well as the purely chance occurrence of minor physical discomforts or subclinical infection may also contribute substantially to the variability in this type of study.

From the data obtained, several patterns are evident which are clearly applicable to the clinical use and evaluation of response to Pyrexal and other endotoxins. A maximal granulocyte increment was obtained at a dose below that caus-

ing appreciable fever and side effects, making it possible to attain this cellular response at a low level of toxicity. The constancy of the peak granulocyte response as the dose was increased beyond this point contrasts with the progressively increasing fever, side effects, and release of immature granulocytes. It is thus apparent that the granulocytic and febrile responses to the pyrogen are separable and do not necessarily involve the same mechanism. Prolongation of the granulocytosis is seen at doses above those needed to produce the maximal cellular response. This may relate either to an increase in the time over which cells are released or, as suggested by Craddock, an increased peripheral survival time of immature granulocytes released in response to leukocytopheresis or other stimuli (18).

It is noteworthy that a significant increase in immature granulocyte count was not observed until a fairly marked febrile response had developed, appearance of these young forms being associated with doses well above those required for maximal granulocytosis. It is not clear why these cells should be released at a time when a large reserve of more mature elements is presumably available. The fact that the appearance of large numbers of immature granulocytes did not increase the total granulocyte count at higher doses is also of interest. It is possible that a qualitative difference exists in the marrow stimulation between the dose that does not evoke an immature granulocyte response and one that causes appreciable numbers of these cells to enter the circulation. Larger doses of endotoxin could also exert a greater destructive effect on peripheral granulocytes, accelerating the turnover rate and thus imposing a greater drain on marrow reserves. A shift to the left accompanies the granulocytosis which is observed during malarial fever therapy and after fevers produced by means of a heat cabinet (19). Fink noted no diminution in the over-all granulocyte response to Pyrexal when the fever was suppressed by acetylsalicylic acid (20).

The initial leukopenia following the injection of endotoxin has been ascribed to alterations in the physical properties of the granulocytes, and perhaps the vascular epithelium, leading to increased sequestration within the capillary bed (reviewed in Ref. 8). Stetson found a correlation between degree of leukopenia and number of sequestered granulocytes in the lung tissue of rabbits injected with endotoxin (21). The recent experiments of Athens and colleagues indicate that there is a shift of cells from the circulating to the marginal granulocyte pool at this time without a change in the total blood granulocyte pool (10). We observed no consistent correlation between dose and degree of granulocytopenia or between degree of granulocytopenia and ensuing granulocytosis, but more frequent counts would be needed for quantitative evaluation of this phase of the response. Possibly the lymphopenia, which was dose related and particularly pronounced after the two highest doses used (Figure 1), is also related to changes in surface properties of the cells and vascular epithelium, differing in time and dose relationship, however, from the changes observed with granulocytes. The lympholytic effect of adrenocortical hormones released in the over-all response to endotoxin must also be considered in this regard (22).

As tolerance to the pyrogenic effect of endotoxin developed on repeated administration, the peak granulocyte increment remained essentially unaltered. This was true in spite of a daily drop in the preinjection granulocyte count. The percentage of immature forms, degree of mononucleocytopenia, and prolongation of granulocytosis also declined on successive days, so that by the sixth day of injection of 5 mμg per kg the entire picture of response was similar to that seen after a single injection of a dose one-tenth that amount. It should be recalled that the total granulocyte increment was stable over the dose range of 0.5 to 5 mμg per kg, and that mature granulocyte release occurred at a considerably lower threshold of endotoxin stimulation than the other parameters of response observed here. The same pattern was followed as tolerance developed in an individual as in the dose-response study of the entire group. Although we found the increment in mature granulocytes to persist essentially unaltered after 8 successive days of endotoxin injections (three subjects), it seems likely that a longer series of injections would result in eventual diminution. Thus, Fukuda and Matsumoto (23) found that the leukocytosis resulting from the intravenous injection of typhoid vaccine in rabbits was greatly decreased by the twentieth consecutive day and

Smith, Alderman and Gillespie (24) noted a diminution in the granulocytosis following intraperitoneal typhoid endotoxin in mice after the last of six injections given over a 2-week period.

A drop in the daily preinjection granulocyte count over the first 4 days occurred in every subject receiving repeated Pyrexal administration. This would not be expected if the marrow reserve and its availability were unlimited. Neither the total number of cells mobilized at each stimulation nor the rate of marrow repletion is known. Smith, Alderman and Cornfield demonstrated a 20 per cent depletion in the nucleated cells of the femoral marrow in mice after endotoxin administration, which persisted for at least 24 hours after injection (25). The drop in peripheral counts presumably reflects a temporary diminution in the marrow granulocytic reserves due to an outflow in excess of the rate of influx from the proliferating pool. The diminution in the release of immature forms with repeated endotoxin administration may be considered evidence that proliferation is not keeping pace with the demand imposed by this stimulus. One should also consider the possibility that endotoxin itself affects the balance between marrow and circulating cells. For instance, inhibition of stem-cell proliferation would lead to depletion of the marrow reserve (see, however, Ref. 28 below); shortening of the maturation time would reduce the immature cell count; and damage to the circulating cells might effect a shortening of their peripheral life span with an over-all increase in rate of capillary bed sequestration or flow into the tissue spaces, or both. The possibility of direct damage to intramedullary granulocytes must also be considered.

Cessation of the downward trend in the preinjection count (and occasional increase in immature forms) toward the end of the series of daily injections may reflect a proliferative stimulus to granulocyte precursors, either as a direct effect of the endotoxin or indirectly as a result of repeated marrow depletion. Windle and Wilcox observed extramedullary hematopoiesis in rabbits receiving a prolonged course of endotoxin injections (26). Smith and co-workers have shown that a single injection of endotoxin prior to or immediately after irradiation in mice and hamsters resulted in a marked increase in bone marrow cellularity over that of noninjected animals within a few days after exposure (27). Recent studies have presented evidence that an increase in mitotic activity of all granulocyte precursor elements occurred after stimulation with pyrogen (28). The evidence that endotoxin stimulation itself can alter bone marrow cellularity may compromise its usefulness as a sequential measure of marrow reserve, particularly in patients receiving myelosuppressive agents or in those having diseases associated with changing levels of myelosuppression.

In the use of endotoxin for test purposes, allowance should be made for the variability introduced by disease and therapy on the reactivity of individuals to this stimulus as well as on marrow function per se. The decreased reactivity to typhoid vaccine exhibited by patients having had malaria has been discussed. Keller and Heilmeyer noted severe side effects when Pyrexal was administered to patients having "splenic tumor" (13) and we encountered hypotension, prolonged chills, and hyperpyrexia in two of our patients having lymphomatous disease when they were given 3 mμg per kg, a dose relatively well tolerated by normal subjects (29). Both of these patients developed an intense immature granulocytosis which may have indicated an alteration in marrow function but which could also have been a reflection of their increased sensitivity to the pyrogen, or perhaps a combination of both. Patients with cirrhosis of the liver have shown both an above average febrile reaction (17) and a subnormal granulocyte response (6, 30). Patients having "hypersplenism" or infection, conditions which may alter granulocyte dynamics, have been noted by Keller (30) and Craddock and colleagues (7) to show inconstant granulocyte responses to endotoxin stimulation. It is obviously important to avoid excessive dosage of endotoxin in attempting to evaluate the marrow granulocyte reserve, but altered physiologic circumstances may make it difficult to predict the optimal test dose in each case.

Functional damage of the reticuloendothelial system induced by colloidal agents such as iron saccharate, trypan blue or Thorotrast greatly increases the sensitivity of experimental animals to the damaging effects of endotoxin (31). Cortisone, which also affects reticuloendothelial system function, has likewise been shown to alter reactivity to endotoxin (31, 32). The effects on the reticuloendothelial system of cytotoxic agents

used in cancer chemotherapy require study in this regard.

There is a number of limiting factors which should be considered before attempting to interpret aberrant febrile and leukocytic response patterns to endotoxin stimulation. These include alterations in reactivity imposed by tolerance, various pathologic states, and certain forms of therapy, as well as changes in granulocyte dynamics relating to the test stimulus. In spite of these limitations the data presented here for normal subjects should provide a useful baseline for evaluating response to endotoxin. In this respect the data have pertinence not only to granulocyte studies, but also to other clinical applications.

SUMMARY

1. Studies of the leukocytic and febrile response to graduated and repeated doses of a purified bacterial endotoxin, Pyrexal, with dosage ranging from 0.05 to 5 mμg per kg body weight, were undertaken in 69 normal volunteers.

2. A maximal rise in total granulocytes was obtained with 0.8 mμg per kg. Higher doses caused a marked shift to the left with prolonged granulocytosis as well as a progressive increase in fever, unpleasant side effects and degree of lymphopenia, but no further change in the total granulocyte increment.

3. With the repeated administration of the pyrogen the daily peak granulocyte increment remained uniform, although fever, side effects and the immature granulocyte increment decreased progressively. Preinjection counts declined steadily over the first few days, suggesting that the test material itself might affect granulocyte dynamics.

4. Data have been obtained that may serve as a baseline for clinical use, provided one takes into account the variability imposed by altered reactivity to endotoxin as well as changes in granulocyte dynamics caused by the agent itself or, particularly in the case of repeated administration, by the reactions which the agent evokes.

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