Comparison of Hematologic and Febrile Response to Endotoxin in Man

By Sheldon M. Wolff, Melvin Rubenstein, John H. Mulholland and David W. Alling

The profound hematologic alterations evoked in experimental animals following endotoxin administration have been considered to play a major role in the pathogenesis of some of the biologic effects produced by these lipopolysaccharides. Marked leukopenia, primarily granulocytopenia, and subsequent granulocytosis as well as transient thrombocytopenia and hypofibrinogenemia have been reported following endotoxin infusion. It has been suggested that a pyrogen released from leukocytes during the granulocytopenic phase is essential for the production of endotoxin fever in experimental animals, but similar extensive studies in man have not been reported. In addition, the recent demonstration of a linear relationship between endotoxin dose and leukocytic response, directs attention to whether a quantitative relationship exists between endotoxin fever and changes in circulating blood cells in man. Since repeated injections render man less susceptible to the febrile effect of endotoxin, a state defined as tolerance, it would be of interest to determine whether or not this diminished febrile effect is also accompanied by decreased leukocytic response.

In this report comparisons of hematologic and febrile responses following endotoxin injection in endotoxin nontolerant and tolerant human beings revealed that there was no correlation between leukocytic and febrile response.

Materials and Methods

Seventeen control volunteers (ages 19-44), 8 patients with familial Mediterranean fever (FMF) (ages 17-40) and 5 patients with other illnesses (ages 15-55) comprised the study group. The latter patients include 2 Salmonella carriers, 1 patient with undiagnosed recurrent fever suspected of being factitious, 1 patient with recurrent urinary tract infection and a 14-year-old boy with periodic hypothalmic discharge. Twenty-two of the 30 subjects were males. Lipexal, the endotoxin of Salmonella abortus equi, was used throughout the studies. After dilution with sterile pyrogen-free physiologic saline the endotoxin was administered in 0.5 or 2.0 mg/kg doses intravenously between 8 and 9 a.m. Rectal temperatures (C) were taken every half hour for 1 hour prior to and 7 hours after endotoxin injection. Endotoxin was not administered if the patient's base-line temperature was greater than 37.5 C. Febrile responses were plotted on 1 x 1 inch standard graph paper (Keuffel and Esser 12-5265) where 1 hour and 1 degree each equalled 1 inch. The area (cm²) under a 7-hour fever curve, beginning at the time of endotoxin injection, was measured with a compensating polar planimeter (Keuffel and

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Esser no. 4242) and designated as the fever index (F1). Subjects were permitted fluid and food as desired and remained in bed during the study. When the same subjects were studied more than once, a minimum of 2 weeks elapsed between experiments. Sterile, pyrogen-free needles and syringes were used throughout.

Blood drawn before and hourly after endotoxin injection was prevented from clotting with sodium versenate. Total white blood cell counts were done in a Coulter electronic particle counter. 100-cell differential counts were performed on smears stained with Wright's-Giemsa stain; platelets were counted by phase contrast microscopy. Plasma fibrinogen levels were determined after separation as fibrin by clotting and nitrogen was measured by the micro-Kjeldahl method.

Studies as outlined above were done on 7 control subjects (4 normal, 3 FMF's) following the intravenous administration of sterile pyrogen-free physiologic saline; subjects and ward personnel were unaware that saline, rather than endotoxin, had been administered.

Identical studies were performed on 8 normal volunteers following the induction of endotoxin tolerance. In 6 of 8 tolerant subjects the amount of endotoxin was increased daily to a maximum dose of 0.25 μg, which was continued until febrile tolerance was consistently demonstrated. These 6 subjects received from 17 to 24 daily injections of Lipexal. The other 2 volunteers received a daily dose of 2 μg./Kg. for 12 days.

In the presentation of results total granulocyte counts include eosinophils and basophils as well as young and mature polymorphonuclear leukocytes. Similarly, the total mononuclear count is comprised of both lymphocytes and monocytes.

RESULTS

Control Studies

Following saline injection granulocytosis began by the fourth and persisted through the seventh hour (fig. 1). Maximum granulocytosis occurred at the fifth hour with a mean increase of 1800 cells per mm. There were only minor changes in mononuclear cell count (fig. 1). The average F1 of the 7 saline control subjects was 8 cm.2, which probably represents the normal daytime pattern of temperature.

Comparisons of Hematologic Response in Normals, FMF Patients and Subjects with Other Illnesses

The mean granulocyte changes of 7 control volunteers, 5 FMF patients, and 5 patients with other illnesses, all of whom received 0.5 mμg./Kg. of Lipexal, were compared. The corresponding mean hourly changes in the granulocytes for the subjects in each diagnostic category were not significantly different. The same was true of the mononuclear cell changes, and also changes in platelet counts and fibrinogen levels. Therefore, in presenting the data no distinction will henceforth be made between these groups.

Effect of Endotoxin Dose on Leukocyte Response

The mean granulocytic and mononuclear responses for 17 subjects receiving 0.5 mμg./Kg. and seven 2.0 mμg./Kg. of Lipexal are depicted in figure 2. Although during the first 2 hours, 15 of the 24 subjects in both dosage groups exhibited slight granulocytopenia, the average neutropenia was not statistically significant (t-test). Mononucleocytopenia, however, occurred in all 24 individuals and accounted for the consistently observed leukopenia during the first or second hour (fig. 2). The decrease in mono-
Fig. 1.—Leukocyte changes in 7 subjects receiving saline. Saline injected at zero time. Data expressed as mean changes from the base line. $\bullet$ = granulocytes; $\Delta$ = mononuclear cells.

nuclear cells was largely due to a fall in lymphocytes, but in some individuals there was also a concomitant drop in monocytes. The decrease in mononuclear cell counts associated with the 2 doses of endotoxin were not significantly different (fig. 2). A maximum granulocytosis of 6300 cells/mm$^3$ was noted at 4 hours in the 0.5 m$\mu$g./Kg. dose group, while the subjects who received 2.0 m$\mu$g./Kg. had a maximum elevation of 7100 cells/mm$^3$ at 5 hours (fig. 2). Although at each hour the magnitudes of the granulocyte counts in both groups were not significantly different, and the slopes of the curves up to the fourth hour were likewise similar, in subjects receiving the higher dose the granulocyte count continued to rise for 1 hour longer ($p < 0.01$, t-test).

In order to dispose of the possibility that significant granulocytopenia occurred earlier than the first hour, 4 normal subjects were given 0.5 m$\mu$g./Kg. of Lipexal and white blood cell counts were done every 15 minutes during the first hour. The results of these studies are presented in table 1. In these 4 subjects, no significant granulocytopenia was observed at any time (Von Neumann’s ratio test, $p > 0.20$). When a fall in the total white blood cell count was noted, it was accounted for by a decrease in mononuclear cell count in all subjects.

Comparison of Leukocyte Response to Magnitude of Fever

Results of studies in 24 nontolerant individuals were grouped according to fever index; the first group was comprised of 8 subjects with FI 11–23;
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As stated above, a decrease in mononuclear cells was observed in all 24 subjects. Fig. 2.—Effect of endotoxin dose on leukocyte response. Endotoxin injected at zero time. Data expressed as mean changes from the base line. • --- • = granulocytes, 2.0 mg./Kg. (7 subjects); △ --- △ = granulocytes, 0.5 mg./Kg. (14 subjects); • --- • = mononuclear cells, 2.0 mg./Kg.; △ --- △ = mononuclear cells 0.5 mg./Kg.

the second of 8 subjects with FI 24-32; the third of 8 subjects with FI 33-71. As previously noted, granulocytopenia was not significant, but in each group the consistent fall in mononuclear cell counts accounted for the leukopenia observed during the first 2 hours (figs. 3 and 4). Granulocytosis noted in the 3 FI categories was maximal at 4 hours and comparison of the granulocytic responses revealed no significant differences (fig. 3). The total lack of correlation between febrile response and granulocytopenia or granulocytosis is further demonstrated in figure 5 in which increases and maximum decreases of granulocytes for individual subjects are plotted against FI. It is noteworthy that subjects with the least granulocytopenia tended subsequently to have the most granulocytosis and, conversely, subjects with the most granulocytopenia had the least granulocytosis (p < .01, rank correlation test).

As stated above, a decrease in mononuclear cells was observed in all 24...
Table 1.—Granulocyte Response to Endotoxin

<table>
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<th>Time (min.)</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
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<th>120</th>
<th>240</th>
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<td>3300</td>
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<tr>
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<td>9830</td>
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<tr>
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<td>3360</td>
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<td>4140</td>
<td>3790</td>
<td>3410</td>
<td>3810</td>
<td>10430</td>
</tr>
</tbody>
</table>

*Granulocytes/mm³.

subjects; but, as was the case with granulocytes, there was no correlation with the magnitude of the febrile response (figs. 4 and 6).

Effect of Endotoxin Tolerance on Leukocyte Response

The granulocytic and mononuclear cell responses in tolerant individuals as compared to nontolerant are shown in figures 3 and 4. The granulocytosis, as well as the decrease in mononuclear cells of tolerant subjects, are strikingly similar to the changes noted in nontolerant individuals, despite the fact that the febrile responses of the tolerant subjects were considerably reduced when compared to those of nontolerant recipients. The maximal changes in mononuclear cell counts of the tolerant and the nontolerant groups were noted between the third and fourth and sixth and seventh hours; there was no significant difference between these groups.

It has been recently reported that during the establishment of endotoxin tolerance in man the base-line granulocyte count decreased. Comparison of the base-line granulocyte counts of 7 subjects before and after the establishment of tolerance revealed a mean decrease of 557/mm³ which was not significant (p > .20, t-test).

Effect of Endotoxin on Platelet Counts and Fibrinogen Levels

Evaluation of the mean platelet counts and fibrinogen levels of all subjects studied revealed no consistent changes associated with the dose of endotoxin, the magnitude of febrile response or the status of the subject (tolerant or nontolerant).

Discussion

Most of the previous studies of leukocytic response to endotoxin in man have been primarily concerned with the evaluation of bone marrow reserve. In these studies no emphasis was placed on febrile response and, indeed, in one study fever was suppressed by aspirin. Recently Mechanic and co-workers reported that the leukocytic response to endotoxin is proportional to the dose given. These same authors found that febrile response was also proportional to the dose of endotoxin administered. However, we are unaware of any attempt to compare the magnitude of the febrile response to hematologic changes. In addition, no information is available on the effect of endotoxin on platelets in vivo in studies involving human subjects.
Fig. 3.—Granulocyte response according to magnitude of fever. Data expressed as mean changes from the base line. — - - = FI 11–23 (8 subjects); — = FI 24–32 (8 subjects); — — — = FI 33–71 (8 subjects); — — — — = tolerant (8 subjects); — — — — — = saline controls (7 subjects). See text.

Fig. 4.—Mononuclear cell response according to magnitude of fever. — - - = FI 11–23 (8 subjects); ——— — = FI 24–32 (8 subjects); — — — = FI 33–71 (8 subjects); — — — — — = tolerant (8 subjects); ——— — — — = saline controls (7 subjects). See text.
Fig. 5.—Maximum increase (Δ) and decrease (·) of granulocytes following endotoxin in 24 nontolerant subjects plotted according to fever index. The plotted regression lines for maximum increase and maximum decrease have slopes of +0.024 and −0.007, respectively.

Fig. 6.—Maximum decrease in mononuclear cells following endotoxin in 24 nontolerant subjects plotted according to fever index. The regression line has a slope of +0.010.
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Tolerance to the pyrogenic effect of endotoxin has been noted to be accompanied by an alteration in response to many of the other biologic effects of these lipopolysaccharides. For example, the prevention of the Shwartzman reaction, diminished tumor necrosis, and decreased lethality have been observed in tolerant experimental animals. In this regard, Olitzki, Avinery and Bendersky noted that with repeated endotoxin injections there was a progressive diminution in the degree of leukopenia accompanying each injection. In addition, Fukuda and Matsumoto described a decrease in the level of peak leukocytosis in rabbits following 20 days of typhoid vaccine administration. In contradistinction to these observations, it has been reported that the magnitude of peak granulocyte counts did not change in man following 8 daily injections of Lipexal. However, the degree of mononuclear cell fall and the duration of the granulocytosis diminished such that by the sixth daily injection the response was equivalent to that induced by one-tenth the dose of Lipexal in nontolerant subjects. On the strength of these latter observations the authors hypothesized that the peak granulocyte count might diminish if a longer series of endotoxin injections were employed. The present findings do not support this prediction, since no fall in peak granulocyte count was noted in subjects who had received endotoxin for a minimum of 12 days and in some instances for as long as 24. Furthermore, the degree of mononuclear cell fall and the duration of granulocytosis were unaffected by the extended period of repeated daily injections of endotoxin. Thus the development of febrile tolerance to endotoxin was unaccompanied by diminution of mononuclear cell fall or granulocytosis.

It has been suggested that the daily leukocytosis during the induction of endotoxin tolerance results in depletion of bone marrow reserve. This hypothesis was based on the observation of a fall in daily preinjection granulocyte count during the first 4 days of endotoxin injections; the diminished baseline count persisted for the 8 days of the study. Although, in our studies, hematologic response of tolerant subjects was determined on only the first and last days of endotoxin administration, the pretolerant and tolerant baseline granulocyte counts were not significantly different. Even though granulocytopenia did not invariably occur after administration of endotoxin and was not statistically significant, there was a highly significant inverse relationship between the height of the granulocytosis and the depth of the granulocytopenia. Using a radioactive disopropyl fluorophosphate-labeled leukocyte technic, Athens and associates showed that the neutropenia following endotoxin administration in man was due to a shift of granulocytes from the circulating to the marginal pool, while the subsequent granulocytosis was related to an increase of the total pool. In subjects who did not acquire neutropenia, the total blood granulocyte pool increased simultaneously with the shift from the circulating to the marginal pool and obscured the neutropenia. The present data are consistent with this mechanism in that the greater the granulocytosis occurring as a result of endotoxin, the smaller was the neutropenia, thus suggesting
that the vigorous outpouring of granulocytes from the marrow tended to obscure the possible neutropenia.

Although the theory is tenable that endotoxin fever in animals is due to release of an endogenous pyrogen from granulocytes during the granulocytopenic phase, it should be noted that in man there is a marked contrast in hematologic response since the rabbit reacts with a prompt (maximal at 1 hour) and prolonged (up to 3 hours) leukopenia, which was not observed in the present studies. The characteristics of the febrile response are also quite different in man since the classical biphasic temperature curve observed in the rabbit has not been noted in an extensive series of studies in human subjects. In addition, in man there is a longer delay before onset of fever following endotoxin administration than in the rabbit and finally, mechanisms of heat conservation and loss are quite different in these two species.

Among the many biologic and biochemical effects of endotoxin observed in animals, fever has been considered to be one of the most sensitive indicators of reactivity. However, it has been reported that in the rabbit hematologic response is a more sensitive index of endotoxin reactivity than fever, but the validity of this concept has been questioned. Hematologic response, observed in many subjects who experienced little or no fever, emphasizes the greater sensitivity of this variable in man.

Mechanic and co-worker reported that in man changes in both granulocytes and mononuclear cells were proportional to the dose of Lipexal employed. In our studies using the same product, a mononuclear cell fall following endotoxin injection was noted, however, there was no significant difference between the two dosage groups; in addition, the magnitude of rise in granulocyte count was the same. However, at the higher dose level the rising granulocyte count was sustained for a longer period of time (fig. 2), thus confirming the dose-response relationship previously described.

Data in control subjects, injected with saline, support the observations reported almost 40 years ago that an afternoon rise occurs in total white blood cell and granulocyte counts of untreated normal human beings. Leukocytosis, similar in magnitude to that observed following saline (ca 2000 cells), has been attributed to endotoxin. Although a granulocytosis of this magnitude probably is indicative of a functioning bone marrow, the stimulus for this response is not necessarily endotoxin. Therefore, studies showing minimal leukocyte changes should include a comparison of the experimental data with that of controls during a period of observation.

In patients with familial Mediterranean fever, episodes of fever and peritonitis, characteristic of this illness, have been shown to alter endotoxin tolerance. Despite this altered febrile response to endotoxin administration, the similarity of the hematologic responses of these patients and of normal subjects suggests that the various effects of endotoxin reactivity are independent of each other.

Although the dosages of endotoxin used in these studies were sufficient to elicit marked leukocytic alterations and in some cases moderate to severe
fever, thrombocytopenia and alterations in fibrinogen levels were not noted. These results are at variance with those reported in experimental animals, and with the report of Shulman who observed hypofibrinogenemia after the injection of typhoid vaccine in man. These discrepancies might be explained by difference in species, endotoxin preparation or dose administered.

It has been suggested that the leukocytosis and the mononuclear cell fall following endotoxin is attributable to the release of adrenocortical hormones, which are known to produce such effects. However, it has been pointed out that the evidence supporting this suggestion is inconclusive. Following the administration of single injections of endotoxin, a linear relationship exists between the maximum fever and the level of plasma 17-hydroxycorticoids in man. Based on this observation it might be anticipated that a similar relationship would hold for fever and hematologic response, a relationship which our data does not support. In addition, some subjects exhibited marked hematologic changes, despite minimal fever and little or no change in plasma cortisol. Furthermore, recent studies in our laboratory have demonstrated that tolerant subjects have a significantly smaller rise in plasma cortisol than nontolerant when challenged with endotoxin, whereas, as reported herein, leukocytic responsiveness is undiminished. Among the remaining explanations for endotoxin-induced hematologic changes are a direct effect of these lipopolysaccharides on hematopoietic tissues or mediation by humoral factors as yet unknown.

SUMMARY

The infusion of endotoxin in man is followed by a fall in mononuclear cell count and subsequent granulocytosis. Significant granulocytopenia was not observed, nor were changes in plasma fibrinogen levels or platelet counts noted. Although a correlation was demonstrated between dose of endotoxin administered and hematologic response, there was no correlation between the magnitude of fever and hematologic changes. Hematologic responsiveness was unchanged following the development of febrile tolerance to endotoxin.

SUMMARIO IN INTERLINGUA

In le homine le infusion de endotoxina es sequite de un declino in le numeratio de cellulas mononucleari, con subsequente granulocytosis. Grados significative de granulocytopenia non esseva observate, e le mesme valeva pro alterationes del concentration de fibrinogeno plasmatic e del numeratio de placchettal. Ben que un correlation esseva demonstrate inter le dose del endotoxina administrate e le responsa hematologic nulle correlation esseva presente inter le magnitude de febre e le alterationes hematologic. Le responsivitate hematologic monstrava nulle alteration post le disveloppamento de un tolera- tion febril pro le endotoxina.

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