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Comparative Study of *Escherichia coli* Endotoxin, Hydrocortisone and Beljanski Leukocyte Restorer Activity in Cyclophosphamide-Treated Rabbits[1] (41296)

MICHEL PLAWECKI AND MIRKO BELJANSKI

Faculté de Pharmacie, Service de Pharmacodynamie, Châtenay-Malabry 92290, France

Abstract. The leukopoietic activity of Beljanski leukocyte restorer(s) (BLR(s)) (RNA-fragments obtained from *Escherichia coli* rRNA), *E. coli* endotoxin, and hydrocortisone administered iv was compared in rabbits treated daily with high doses of cyclophosphamide (CP), a drug which decreases the circulating leukocyte count. Results showed that endotoxin and hydrocortisone responses, characterized essentially by granulocytosis, occurred 3–24 hr following drug injection and disappeared thereafter. Maximal BLR leukocytosis occurred at the 48th hr and remained, even during daily CP administration, within physiological limits for 3–5 days. No tolerance was induced with by administration of BLR in normal rabbits, even after 11 iv injections, implying no depletion of bone marrow cells. In contrast, repeated endotoxin injections led to febrile and leukocytic tolerance. In addition, BLR induced normal leukocytosis and a biphasic fever response in endotoxin-tolerant animals. When BLR and endotoxin were mixed and administered every day to rabbits, the animals became tolerant to endotoxin but gave a normal fever and leukocyte response to BLR, even after the 14th injection. The imbalance induced by CP administration in the granulocyte/lymphocyte ratio may be corrected following an injection of BLR. These data showed that the physiological activity of BLR in leukopoiesis was clearly distinguishable from that manifested by *E. coli* endotoxin and in the second instance by hydrocortisone.

We have described elsewhere (1, 2) the physiological effect exhibited by RNA-fragments[2] designated BLRs which restore normal leukocyte and platelet counts in rabbits where both types of blood cells had been decreased by various anticancer drugs (3, 4). This substance was prepared by the controlled degradation of purified *Escherichia coli* rRNA by pancreatic ribonuclease (5). Because of BLR's bacterial origin and because of its adrenal localization in rabbits, we now present a comparative study of leukocyte appearance in the presence of either BLR, endotoxin, or hydrocortisone. The tolerance phenomenon, characteristic of bacterial endotoxins, was also investigated with various BLR preparations.

Materials and Methods. Male New Zealand white rabbits weighing approximately 3.5 kg were used throughout the experiments. Purified *E. coli* endotoxin serotype 0111 B4 (Difco) was a gift from Dr. M. Roumiantzeff, Institut Mérieux. It was injected iv in concentrated doses of 1.7 μg/kg except where otherwise indicated. Hydrocortisone (Roussel-Uclaf, Paris) was used at a concentration of 3.5 mg/kg. Cyclophosphamide (Endoxan, Laboratoires Lucien, Colombes, France) was injected iv in concentrations of 20–30 mg/kg/day. BLR doses were generally 0.3–2 mg/kg.

The preparation of BLR. The preparation of BLR has been previously described (5). Briefly, BLRs were prepared from purified ribosomal *E. coli* RNA (rRNA) (23 S + 16 S) by mild and controlled degradation in the presence of pancreatic ribonuclease and then purified by several chloroform treatments. After dialysis, the resulting RNA-fragments were purine rich; they contained 20–50 nucleotides and the uv absorption spectrum was characteristic of single-stranded ribonucleic acid-type molecules. Their sedimentation coefficient, 2–3 S, was measured by gel electrophoresis.

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[2] *Abbreviations used:* BLR(s), Beljanski leukocyte restorer(s). CP, cyclophosphamide; LPS, lipopolysaccharide; EDTA, ethylendiamidetetraacetic acid; RNA, ribonucleic acid; rRNA, ribosomal RNA; LAL, *Limulus* amoebocyte lysate. RNA-fragments, RNA chains containing 20–30 ribonucleotides.

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Cyclophosphamide treatment. The rabbits (mean leukocyte count 10^4 cells/mm^3) received iv 20–30 mg/kg cyclophosphamide (CP) every day. These doses induced a regular decrease in the circulating leukocyte count which fell from 10^4 to 3.5–5.5 × 10^3 cells/mm^3 by approximately the eighth day. During this period, when the number of circulating leukocytes represented about 50% of the initial leukocyte count, the daily CP doses were always regularly administered. In addition, rabbits received either endotoxin, hydrocortisone, or BLR.

Blood cell estimation. At the indicated intervals venous blood was taken from the marginal vein of the ear, using EDTA as anticoagulant. Leukocyte and red cell counts as well as hematocrit were performed using a Coulter electronic cell counter. After spreading and fixation of cells using the May–Grunwald–Giemsa method, differential leukocyte counts were taken. A minimum of 200 cells was counted. Cells were classified as granulocytes (including neutrophils, eosinophils, and basophils) and lymphocytes.

Pyrogenic effects. The pyrogenic effect of endotoxin and BLR was measured by taking the anal temperature with a thermometer graduated to 0.1°. Rabbits' temperatures were taken 1 hr prior to iv administration of the drug and animals with an anal temperature greater than 40° were discarded. The temperature was checked at designated intervals thereafter. Fever response was measured in normal rabbits as the area under the anal temperature curve for 6 hr after injection. The fever index was calculated by plotting 1 hr for 1 cm on the abscissa and 1° for 1 cm on the ordinate. One square centimeter of area represents 1 unit of fever index.

Results. In a previous paper we demonstrated that BLR prepared from *E. coli* rRNA acted *in vitro* as a primer in the replication of DNA isolated from hematopoietic tissues. When injected iv into CP-treated rabbits, BLR physiologically induced a progressive leukocytosis which reached a maximum level 48 hr after administration (Fig. 1). Bacterial endotoxin (LPS), known to produce a rapid leukocytosis (granulocytosis (6, 7)) a few hours after it has been injected, was administered to rabbits receiving 20–30 mg of CP/kg (iv) daily. Figure 2 illustrates the leukocyte response of various doses of LPS. The concentration of LPS which induces a significant leukocyte response in CP-treated rabbits was about 1.7 μg/kg. The maximum increase in the leukocyte count occurred before the 24th hr following the endotoxin injection,

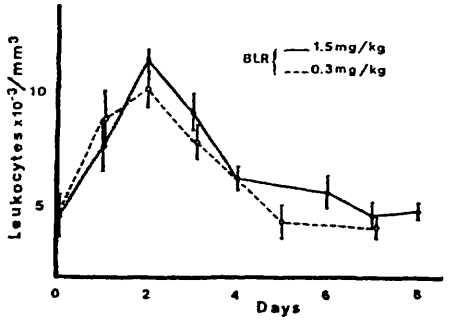


FIG. 1. Leukocyte response of cyclophosphamide-treated rabbits after BLR injection. BLRs were injected iv at 0 hr. Each curve represents the mean values for eight rabbits ± 1 SD. Below a BLR dose of 0.3 mg/kg we did not observe a significant leukocytosis. From 0.3 to 10 mg/kg, BLR restored the normal leukocyte count.

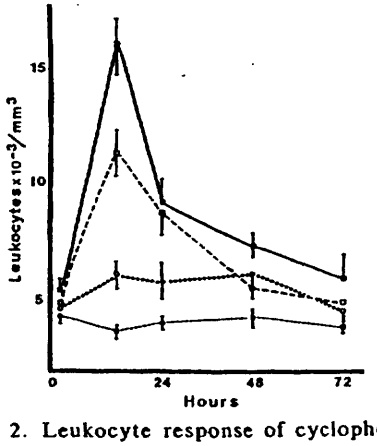


FIG. 2. Leukocyte response of cyclophosphamide-treated rabbits after injection of various LPS concentrations. Each curve represents the mean values for three to six rabbits ± 1 SD. Dark circle: 0.4 μg/kg; open circle: 0.8 μg/kg; open square: 1.7 μg/kg; dark square: 5 μg/kg. (Leukopenia was observed during the first 3 hr following LPS injection. Data are not presented here.)

and was proportional to the concentration injected; with the 5 μg/kg dose the number of leukocytes represented, at the maximum level, about three times the baseline count. This peak of activity decreased rapidly, so that 48 hr after LPS administration the number of white blood cells was practically equal to that observed just before the injection. The leukocyte response induced by LPS in CP-treated rabbits corresponded essentially to a brief granulocytosis (Fig. 3). At the maximum level the number of granulocytes represented 56.2 ± 1.8% of the total white blood cells. There was no significant effect on the lymphocyte pool (Fig. 4).

Figures 3 and 4 show that compared to LPS, BLR increased both types of white blood cells, which reached a maximum level 48 hr after injection. These results clearly indicated that the kinetics of granulocytes and lymphocytes induced by BLR in CP-treated rabbits differed markedly from that induced under the same conditions by *E. coli* LPS.

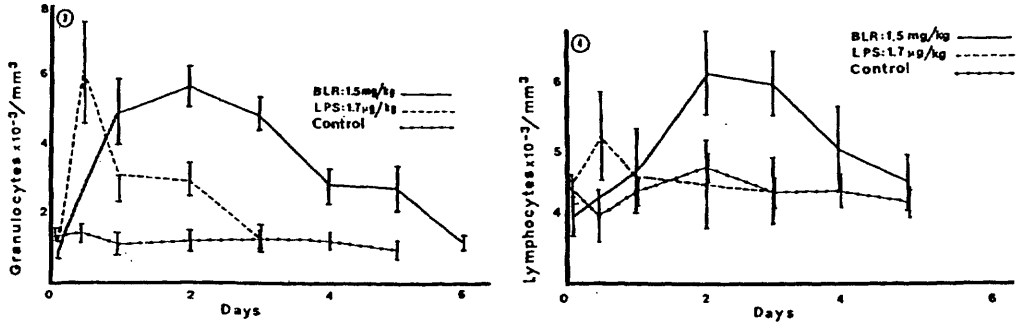
BLR's Schwartzman reaction and LAL test. We first attempted to evaluate the eventual endotoxin content of various BLR preparations by the Schwartzman reaction. BLRs were injected up to 250 μg (id) into five different sites. The rabbits were then challenged 16 hr later with 1 mg/kg iv BLR. None of the many BLR preparations tested gave a positive Schwartzman reaction.

The LAL test is a sensitive assay system used for detection of pyrogenic material in biological preparations (8, 9). It can detect less than 0.25 ng of bacterial endotoxin and gave positive reactions with proteins and polyribonucleotides (10, 11).

In our assays, the results obtained with standardized BLR preparations varied from 3 to 183 ng of endotoxin per milligram of material tested (using various LAL preparations). Poly(A), inactive in leukopoiesis, gave a positive reaction (162 ng/mg) while poly(C) was only weakly reactive (0.8 ng/mg). Poly(I)–poly(C) and poly(A)– poly (U), which are pyrogenic, also produced a positive reaction (11). We should recall that BLRs are purine-rich RNA-fragments (G + A/C + U = 2.32), and that various lysate preparations contained different levels of RNase activity which degraded both purine-rich and pyrimidine polymers, although the latter are only poorly degraded.

Whatever their apparent endotoxin content, BLR injected into CP-treated rabbits invariably induced an increase in leukocytes with the same intensity and kinetics. This induction was far different from the granulopoietic effect of various endotoxin concentrations as shown in Fig. 2.

Tolerance. Tolerance is a well-known property of bacterial endotoxin and can be obtained even with a 10 ng/kg dose (12, 13). Since the LAL test is far from being specific, we developed a comparative assay between BLR and *E. coli* LPS in order to detect possible endotoxin activity in BLR preparations. A single dose of *E. coli* LPS 0111 B4 (1.7 μg/kg) provoked an increase of body temperature in normal rabbits to a maximum of about 1.5°. The two peaks of



FIGS. 3 and 4. Granulocyte and leukocyte increase in cyclophosphamide-treated rabbits after BLR or LPS injections at 0 hr. Each curve represents the mean values for 7 to 10 rabbits ± 1 SD. For control experiment, the rabbits were injected with a physiological saline solution.

TABLE I. FEVER INDEX AFTER REPEATED LPS OR BLR INJECTIONS INTO NORMAL RABBITS

	Number of injections			
	1	4	7	11
LPS	5.4 ± 0.4	3.25 ± 0.3	1.95 ± 0.1	N.D.[a]
BLR	5.0 ± 0.3	6.6 ± 1.5	7.0 ± 1.5	7.4 ± 1.5

Note. Results (area under the fever curve) are mean values of three different experiments ± 1 SD.
[a] Nondetermined.

fever response occurred at 1 and 3 hr after LPS administration.

We observed a progressive decrease of the fever response in rabbits which had received alternate iv LPS injections. Tolerance appeared after the fourth injection and was almost complete after the seventh LPS injection (Table I). It corresponded to the disappearance of the second peak of body temperature. BLRs were comparatively administered iv (1.5 mg/kg). The body temperature reached two peaks, one between the first and second hour, the second between the third and fourth hour. *No tolerance was observed even after the 11th BLR injection.* The febrile response remained constant during these injections and no decrease of body temperature at the third hr was observed (Table I). In this respect, BLRs behaved in the same way as tissue or leukocyte pyrogens (14, 15).

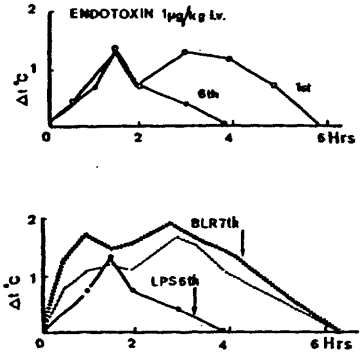


FIG. 5. Mean temperature curves for a group of three normal rabbits after daily injection of 1.7 μg LPS/kg. All the inoculations were performed at 0 hr. Upper plot—open circle: mean fever response after the first LPS injection; dark circle: mean fever response after the sixth LPS injection. Lower plot—(■■■) mean fever response of the above tolerant rabbits which received, on Day 7, 1.5 mg BLR(s)/kg. (– – –) mean fever response of non tolerant rabbits after injection of 1.5 mg BLR/kg. (BLR at 0.03, 1.5, or 3 mg/kg gave the same fever index.)

Figure 5 shows the fever response of rabbits made tolerant by daily injections of LPS. When tolerance was established after the sixth injection, animals were injected 24 hr later with BLR iv. The fever response to BLR injection in endotoxin-tolerant rabbits was identical to that exhibited in nontolerant rabbits. After a BLR injection, rabbits tolerant to *E. coli* endotoxin gave a fever response analogous to that observed in nontolerant rabbits.

In parallel, the leukocytosis induced by LPS injections regularly decreased so that after the seventh injection the leukocyte count was only 31% higher than the baseline count (Table II). Tolerance occurred not only in the body temperature but also in the leukocyte response (16, 17). Repetition of BLR injections did not decrease the ability of rabbits to respond normally to fresh administration of BLR (Table II).

In order to separate the leukocyte responses induced by LPS and BLR, we administered a daily mixture of BLR (1.5 mg/kg) and LPS (1.7 μg/kg) iv to normal rabbits. The leukocyte response induced by this mixture remained practically constant during the daily injections (Fig. 6). After the

TABLE II. LEUKOCYTE INCREASE AFTER REPEATED LPS OR BLR INJECTIONS

	Number of injections			
	1	4	7	11
LPS	159 ± 22	49 ± 10	31 ± 5	N.D.
BLR	121 ± 5	126 ± 16	122 ± 9	120 ± 13

Note. Values represent the percentage of leukocyte increase 16 hr after drug injection over the baseline count. They are mean values of three differents experiments ± 1 SD.

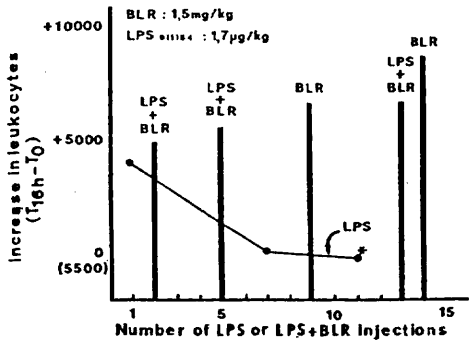


FIG. 6. Number of leukocyte increase over the baseline count 16 hr after challenge. Rabbits were injected daily with a mixture of 1.5 mg of BLR + 1.7 μg of LPS/kg. The columns represent the mean increase in leukocyte count after challenge with BLR + LPS or BLR alone (Days 9 and 14). The curve represents the mean increase in leukocyte count in the same rabbits, after challenge with LPS alone on Days 1 and 7; on Day 11, rabbits were challenged with 5.5 μg of LPS/kg.

7th day, the rabbits were injected with the LPS dose only. The leukocyte-induced response was severely diminished and was only 30% over the baseline count. When three times more LPS was injected (11th injection) no further leukocyte increase was observed. When *the same tolerant* rabbits were injected with BLR doses only, the leukocyte response did not differ from that observed after BLR + LPS injections. The animals challenged with a mixture of BLR + LPS became tolerant to *E. coli* LPS only.

We have shown that labeled BLRs injected iv into normal rabbits are localized not only in spleen and bone marrow cells but to a certain extent in the adrenals. Corticosteroids, including hydrocortisone, produced, as did endotoxin, a transient liberation of granulocytes from the reserve pool (7, 18). We have therefore compared BLRs-induced production of leukocytes with that obtained with hydrocortisone in CP-treated rabbits. The iv injection of 3.5 mg hydrocortisone/kg produced a granulocytosis 3 hr after the drug was given. Thereafter, the leukocyte count decreased rapidly and returned to the level observed just before the injection. The maximum level of granulocytes represented 81.6% of the total circulating white blood cells. A second hydrocortisone administration 48 hr after the first failed to induce the same magnitude of neutrophil leukocytosis, thus implying that the leukocyte reserve pool had been partly depleted by the first hydrocortisone injection.

Discussion. The present studies were undertaken to compare leukocyte count changes after iv administration to rabbits of either endotoxin, hydrocortisone, or BLR. Hydrocortisone, which is widely used in clinical medicine, caused a rapid and transient liberation of granulocytes only (7). Endotoxin also induced a transient liberation of granulocytes into the blood, but after repeated injections, it brought about the appearance of tolerance. Rabbits responded very poorly to a fresh injection. In contrast to these two agents, BLR brought about a rapid increase in both the granulocyte and lymphocyte counts which remained within physiological limits for 3 days. *BLR did not induce tolerance.* With endotoxin, the level of temperature was proportional to the log of LPS injected, while febrile response was not proportional to BLR doses of 0.03, 0.3, and 3 mg/kg. The transient fever response and the absence of tolerance observed after BLR injections closely resembled those described for tissue and leukocyte pyrogens (14) which produced a fever response without inducing tolerance even after several iv doses (15).

In order to obtain a clear separation of both types of activities, we injected rabbits every day (iv) with a mixture of endotoxin plus BLR. After six injections rabbits were challenged either with endotoxin alone or with BLR alone. The results firmly established that rabbits became tolerant only to endotoxin, not to BLR. Tolerant rabbits normally increased their leukocyte count when BLRs were injected.

We attempted to evaluate the amount of endotoxin that could contaminate BLR (made from bacterial rRNA) using different amoebocyte lysate preparations (LAL). It has already been shown that the LAL test was not specific to endotoxin and reacted positively with different polyribonucleotides, proteins, or dithiothreitol (19). We have found that synthetic poly (A) gave a clear positive response while poly (C) was poorly active. BLRs, which are rich in

purine nucleotides, also gave a positive response which varied from one LAL preparation to another. The *Limulus* lysate contains variable amounts of ribonucleases activity which are quite specific for purine nucleotides in the polymer chain and this may explain the variations observed with BLR(s).

The absence of tolerance, the restoration of a normal balance between granulocytes and lymphocytes in cyclophosphamide-treated rabbits, and the restoration of platelets in daunorubicin-treated rabbits (2) indicated that BLRs act on the genesis of stem cells and their derivatives; this was expected from their specific priming effect on *in vitro* DNA synthesis of hematopoietic tissues, and from their localization in those tissues.

From all these results, past and present, BLRs, undoubtedly distinguishable from either endotoxin or corticoid, are new and very promising promoters of hematopoiesis.

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