Leukocyte Counts and Activation in Spontaneously Hypertensive and Normotensive Rats

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The etiology for the progressive organ injury in hypertension is largely speculative. Recent studies have shown that leukocytes play a key role in several cardiovascular diseases. As an initial step toward investigating the role of leukocytes in hypertension, we measured leukocyte counts and spontaneous activation of granulocytes of freshly drawn unseparated blood samples in spontaneously hypertensive rats and in their normotensive counterpart, Wistar-Kyoto rats. The animals were derived from one breeder in the United States and from two breeders in Europe. Total leukocyte counts in young, mature, and old hypertensive rats were 50–100% above the controls. The number of granulocytes in mature and old spontaneously hypertensive rats is more than 100% elevated compared with control rats. In young hypertensive rats the mean granulocyte count was only slightly elevated. The number of spontaneously activated granulocytes, as detected by the nitroblue tetrazolium reduction, increases with age in both species; in mature spontaneously hypertensive rats, it is more than 300% above the values in the controls. Furthermore, in mature hypertensive rats the number of monocytes, activated monocytes, and the lymphocyte count are also significantly elevated over the values in the normotensive controls. It is proposed that these elevated leukocyte counts may constitute an enhanced risk for organ injury in the spontaneously hypertensive rat. (Hypertension 1991;17:323–330)

One of the important unresolved issues in hypertension research is the cause of the enhanced organ injury that accompanies this syndrome. The focus of previous investigations centered mostly around the mechanisms responsible for the chronically elevated blood pressure. Comparatively few proposals have been advanced to explore the mechanisms by which organ injury may be mediated.

Evidence accumulating in the last decade suggests that leukocytes not only serve a beneficial immunological function but are directly involved in the etiology of cardiovascular disease. Tissue injury involving leukocytes has been demonstrated in ischemia and reperfusion injury in the heart, skeletal muscle, and intestine, during shock, in atherosclerosis, and in other diseases. Circulating leukocytes, in spite of their small numbers compared with red blood cells, have a substantial influence on the hemodynamics in the microcirculation. Leukocytes, especially granulocytes and monocytes, are capable of producing oxygen free radicals and mediators of proteolytic tissue degradation. Such evidence suggests that the organ injury during chronic hypertension may in part be associated with the activity of the leukocytes in the circulation.

We report on our initial findings in an ongoing investigation of circulating leukocytes in hypertension using the spontaneously hypertensive rat (SHR) experimental model and its normotensive counterpart, the Wistar-Kyoto (WKY) rat. Counts were made of leukocytes and their subfractions in central arterial blood samples together with specific forms of spontaneous leukocyte activation from fresh unseparated blood samples and were correlated with central hemodynamic parameters. The study was carried out in part with animals from breeders in the United States and in...
Europe to circumvent uncertainties related to local and seasonal variations in leukocyte counts.

Methods

SHR and WKY rats were obtained at different ages from Charles River Breeding Laboratories, Wilmington, Mass., Mollegard Breeding Centre Ltd., Eiby, Denmark, and Ivanova Breeding, Kisslegg, Bavaria, FRG. The European group of animals was subjected to a slightly different protocol than their US counterparts. The two groups are reported separately.

The European animals weighed between 250 and 350 g and were between 15 and 18 weeks old. They were fed with commercial pellets (Altronium 1314, Altromin GmbH, Lage, FRG) and water ad libitum. Three measurements of systolic blood pressure in the unanesthetized rats were made with the tail-cuff technique immediately before blood collection. Blood samples were taken by means of heart puncture with an 18-gauge needle after ether anesthesia using EDTA potassium (1 mg/ml) as anticoagulant. The total leukocyte count (number of cells per cubic millimeter=cells/mm³) was measured with a Coulter Counter (model ZF, Coulter Electronics GbmH, Krefeld, FRG). These studies were carried out at different seasons of the year.

The US rats were between 3 weeks and 58 weeks old and fed with standard rat chow and water ad libitum. The animals were maintained under close veterinary supervision, and no animal in the study had overt signs of infection. For the purpose of blood pressure measurements and blood sample withdrawal a femoral catheter was inserted under local anesthesia (4% xylocaine s.c., Antra Pharm., Westborough, Mass.). The animal procedures were previously reviewed and approved by the University of California, San Diego animal subjects committee.

The blood pressure was read continuously via a transducer, and the analog signal was recorded continuously into a laboratory computer that served to detect systolic, diastolic, mean pressure (time averaged), and heart rate. Unless stated otherwise, 0.5 ml arterial blood samples were taken immediately after cannulation to minimize a possible redistribution of circulating leukocytes.

To explore the effects of general anesthesia on circulating white cell count, three rat subgroups were formed and given each one of the following anesthetic agents: 1) sodium pentobarbital (50 mg/kg body wt), 2) chloralose/urethane (1%/14% mixture, 0.6 ml/100 g body wt), and 3) althesin (4.5 mg/kg initial dose followed by 7.5 mg/kg/hr continuous femoral infusion, Glaxovet, Boronia, Australia). Blood samples were taken immediately before and about 30 minutes after administration of the anesthetic agent. The blood samples were drawn into heparin as anticoagulant (about 10 units/10 ml) and processed within 10 minutes. The following measurements were obtained: 1) total white blood cell count by use of a hemocytometer (Fisher Scientific, Fair Lawn, N.J.); 2) differential leukocyte count for granulocytes, lymphocytes, and monocytes from a blood smear; and 3) activation of neutrophils and monocytes by the reduction of nitroblue tetrazolium reduction (NBT test).

The NBT test is a measure for spontaneous superoxide formation in neutrophils and monocytes and was carried out according to the method of Park et al15 as modified by Barroso-Aranda and Schmid-Schönbein.9 Briefly, 0.1 ml of freshly drawn, unseparated arterial blood was immediately transferred into a cleaned (with endotoxin detergent, Sigma Chemical Co., St. Louis), siliconized concave microslide and mixed with an equal amount of 0.1% NBT solution. Blood cell separation was avoided since it can severely influence spontaneous activation. The blood-NBT mixture was incubated at 37°C for 30 minutes followed by 15 minutes at room temperature (about 22°C). After gentle stirring, cover slip smears were made and stained with Wright's stain and 100 neutrophils and 30 monocytes were counted at x 100 oil objective magnification. NBT-positive neutrophils or monocytes showed a stippled cytoplasmic distribution of formazan or a single dense formazan deposit, larger than the cell granules. Blood samples were collected routinely in the morning before 10 AM, and both groups were investigated over 2 consecutive months to avoid daily and seasonal variations in NBT-positive neutrophil counts, which had been previously noted in normotensive animals.9

Statistical comparison of mean values between the normotensive and hypertensive values was carried out by the Student's t test using a BMDP statistical software program (Berkeley, Calif., University of California Press, 1985). Comparison of median values for asymmetric distributions was carried out with the Mann-Whitney test. Values of p<0.05 were considered statistically significant. Linear correlations between paired groups of measurements were expressed in terms of the correlation coefficient r, the probability p, and the number of animals n.

Results

Blood Pressure

The blood pressures of all SHR were significantly elevated over those of WKY rats at any age between 8 and 58 weeks. Mean values for the mature Charles River rats and systolic values for the European rats are listed in Table 1. Mean blood pressure levels of freshly weaned SHR (about 3–4 weeks old), as measured under local anesthesia, are also elevated (79±11 mm Hg in WKY rats [n=12] versus 105±9 mm Hg in SHR [n=13], p<0.05). Systolic and diastolic values in the Charles River SHR were significantly elevated at all ages.

Hematocrit

Hematocrits in SHR ranged between 30% and 48% (n=23) and between 28% and 44% in WKY rats (n=14). Their mean values were not significantly different. There was a positive correlation between the
increase in age and the hematocrit in both strains (r=0.53, p=0.008, n=23 for SHR; r=0.46, p=0.009, n=14 for WKY rats) in the range between 6 weeks and 28 weeks. The least-squares linear regression lines for hematocrit as a function of age (in weeks) were

H=(35+0.051*A)

for SHR, and

H=(34+0.032*A)

for the WKY rats, where H is hematocrit and A is age.

**General Anesthesia and Leukocyte Count**

One of the experimental limitations for a realistic measurement of the leukocyte count is that general anesthesia has been found to lead to a rapid fall in circulating leukocytes. Figure 1 illustrates the situation in terms of the total leukocyte count. In this group of WKY rats and SHR, an arterial sample was taken immediately before and about 30 minutes after intra-arterial administration of nembutal. In each animal the arterial leukocyte count fell, on average by about 23% (n=4) below the count before general anesthesia. At the same time the average central blood pressure fell by 15–20 mm Hg. After alfathesin anesthesia, the circulating leukocyte count fell on average by 23% (n=3) together with a pressure reduction of between 10 and 15 mm Hg. After general anesthesia with chloralose/urethane, the reduction was more severe, 47% for the leukocyte count (n=3) and a mean pressure drop between 30 and 40 mm Hg. In all three cases the pressure reduction and the drop in leukocyte count occurred over the same period of time. SHR and WKY rat strains showed similar trends in this respect. In the present investigation, all cell counts were carried out on blood samples taken during local anesthesia and, in the case of the animals investigated in Europe, under ether anesthesia followed immediately by a heart puncture.

**Total Leukocyte Count**

Table 1 shows a summary of the arterial leukocyte counts from the three different breeders in mature animals between 15 and 28 weeks of age. The leukocyte count in the SHR is almost twice as high as in the controls. These measurements were carried out randomly at different times of the day and at different seasons, and they are in close agreement with a previous measurement in SHR from another breeder in Germany (Bohringer AG, Mannheim, FRG). A trend for the leukocyte count to be elevated in the SHR can be observed over all ages of the rats (Figure 2). It is noteworthy that the total leukocyte count in the freshly weaned SHR is already about 40% higher than counts in the WKY rats at a stage when mean blood pressure is beginning to be elevated. In older SHR, the rise in total leukocyte count is even more pronounced than in normotensive rats. Inspection of individual histograms in age-matched groups of SHR and WKY rats shows little overlap in leukocyte counts, especially when blood samples were drawn under local anesthesia rather than after general anesthesia.

**Differential Leukocyte Counts**

Two subgroups of leukocytes were measured at different ages, neutrophils and combined lympho-
cyte/monocytes (Figure 3). Although the combined lymphocyte/monocyte count does not show a significant trend with age in either strain, the values in SHR are substantially higher at all ages (Figure 3, bottom panel). The average neutrophil counts are similar in the young animals, but they tend to increase with age in the SHR (Figure 3, top panel). At a mature age, the average values of monocyte and lymphocyte counts are both elevated in the hypertensives (Figure 4).

**Neutrophil and Monocyte Activation**

Spontaneous neutrophil and monocyte activation, as detected by the NBT test, was observed in all rats irrespective of age. Typically in our population of rats between 2% and 15% of the neutrophils are NBT-positive; individual values may be higher. Figure 5, top panel, shows a histogram of the NBT-positive neutrophil count (cells/mm$^3$) in the mature SHR and WKY rats. In both strains, the distribution of NBT-positive neutrophils is asymmetric, and their median and their mean values are different. Especially notable is the trend in the mature SHR for activated neutrophil counts to be above 400 cells/mm$^3$, levels that are rarely encountered in normotensive rats of any strain, unless clinical signs of infection are present. Both strains show an increased incidence of neutrophil activation with age above the relatively low values present at a young age (Figure 6). The difference in neutrophil activation between SHR and WKY rats is statistically significant only in mature animals on the basis of the current sample size, although there is a trend in SHR toward higher...
values in both the young and the aged group. The trend in the NBT-positive monocyte count is similar (Figure 5, bottom panel) with threefold higher mean value in mature SHR.

**Correlations**

Our data set makes it possible to explore a number of correlations. At this early stage of the investigation such correlations may serve as guidelines for exploration of mechanisms, since correlations by themselves do not establish cause and effect relations. We will thus limit our presentation to a selected set of results in the mature animals.

The circulating leukocyte counts and mean arterial blood pressure, measured at the time of blood sample collection, show a positive correlation in WKY rats ($r=0.65$, $p=0.007$, $n=15$). No correlation exists in SHR ($r=0.23$, $p=0.38$, $n=14$), although in the combined group the correlation is significant ($r=0.64$, $p<0.001$, $n=29$). A positive correlation was also detected in WKY rats between diastolic pressure and leukocyte count ($r=0.58$, $p=0.025$, $n=15$) but not for systolic pressure.

There was a positive correlation between the combined lymphocyte/monocyte count and mean blood pressure in WKY rats ($r=0.62$, $p=0.012$, $n=15$) but only a marginal correlation in the SHR ($r=0.4$, $p=0.012$, $n=16$). Here again the data for the combined group of SHR and WKY rats showed a positive correlation ($r=0.57$, $p<0.001$, $n=31$). The neutrophil count for either SHR or WKY rats showed no correlation with mean blood pressure, although the same correlation was again significant ($r=0.64$, $p<0.05$, $n=31$) when the data for the two groups were combined. The correlations between the diastolic blood pressure and the leukocyte subgroups were almost identical to those found for the mean pressure above but not for the systolic pressure.

The number of NBT-positive neutrophils was found to show a weak correlation with mean blood pressure only when the data for the two strains were combined ($r=0.36$, $p=0.057$, $n=28$). A similar correlation test with diastolic pressure gave almost identical results. There was no detectable correlation with systolic pressure.

Heart rate at the time of blood sample collection and the NBT-positive neutrophil count in WKY rats showed a strong correlation ($r=0.63$, $p=0.015$, $n=14$) and also in the combined group of WKY rats and SHR ($r=0.48$, $p=0.008$, $n=28$) but not the SHR strain itself.

**Discussion**

**Mechanisms of Hypertension**

In a series of recent micropressure measurements in several rat skeletal muscles, it was shown that about 80% of the overall arterial pressure reduction occurs in the microcirculation distal to the smaller artery arterioles, irrespective of whether the animals are hypertensive (SHR) or normotensive (WKY). The pressure reduction in central arteries feeding skeletal muscle is lower than was presumed previously, and it is a feature that appears to be independent of the choice of skeletal muscle. At issue are the mechanisms that may elevate the hemodynamic resistance in the microcirculation of SHR.
Several proposals have been made in this respect, for example, arterial wall hypertrophy,\textsuperscript{19} which could not be confirmed in most vascular regions of the SHR,\textsuperscript{20} an elevated tone in the smooth muscle of the arterioles,\textsuperscript{21} and structural modifications in the microvascular network.\textsuperscript{22,23} One of the shortcomings of these proposals has been the failure to provide a link between such vascular modifications and the basic complication in the hypertensive animals (i.e., an accelerated organ injury). It is this uncertainty that led us to the current exploration.

**Leukocyte Count as Cardiovascular Risk Factor**

Traditionally an elevated leukocyte count has been regarded as a clinical sign of infection. During the last decade, however, it has become apparent that an elevated leukocyte count also constitutes a risk factor for cardiovascular disease (for reviews, see References 13 and 24). It is in this context that the current finding of elevated leukocyte counts in SHR brings to the fore provocative questions about the role of inflammatory and immune cells in genetic hypertension. In view of their role as mediators of tissue injury, granulocytes and monocytes may contribute to the increased risk for cardiovascular complications in SHR and possibly also in other forms of hypertension. The current data set suggests that this fact is not simply the manifestation of an inflammatory response in the traditional sense. The fact that young animals already display an elevated circulating leukocyte count at a time when the blood pressure is beginning to be elevated suggests the two phenomena may be coupled to a similar or possibly to a common genetic defect in the SHR.

Leukocytes have the potential to contribute in a number of ways to the arterial hypertension per se. The presence of leukocytes has recently been shown to impose in skeletal muscle a substantially higher capillary resistance than was recognized in the past.\textsuperscript{25} Their mode of action in this regard may be twofold: one leads to a constrictor response of vascular smooth muscle via the release of superoxide, which may inactivate the endothelium-derived relaxing factor (nitrous oxide)\textsuperscript{26} or diminish prostacyclin release\textsuperscript{27} or the possible release of a vasoconstrictor peptide;\textsuperscript{28} the other exerts a direct effect on the capillary resistance by virtue of the high stiffness of the leukocyte cytoplasm and the substantial deformation required to transport these cells along the capillaries of many organs.\textsuperscript{25} Each of these possibilities requires further investigation in hypertension.

**Leukocyte Properties in Hypertensives**

No obvious differences in morphology of leukocytes in SHR compared with normotensive rats have been reported under intravital conditions,\textsuperscript{29} although a detailed comparison with the WKY rat strain has not been carried out. In humans, there is considerable evidence suggesting that leukocytes of essential hypertensive individuals have an elevated Na\textsuperscript{+} content in the cytoplasm\textsuperscript{30} due to a lowered (ouabain-sensitive) Na\textsuperscript{+} efflux rate constant that perhaps is mediated by a circulating Na\textsuperscript{+},K\textsuperscript{+}-ATPase inhibitor.\textsuperscript{31} Although such an abnormality may not be unique to hypertension,\textsuperscript{32} it may represent a potential risk factor favoring spontaneous leukocyte activation in the circulation, superoxide formation, adhesion to the endothelium, and rapid progressive organ injury. In this regard, mention should be made of the observations of Ananchenko and his coworkers,\textsuperscript{33,34} who also reported an increased NBT-positive neutrophil count in human hypertensive patients, which was correlated with a loss of immunocompetent cells. The degree of enhanced neutrophil activation is comparable with the situation in the SHR. Human hypertensive patients have been reported to exhibit a decreased activity of cytosolic and mitochondrial superoxide dismutase in polymorphonuclear leukocytes and an enhanced rate of lipid peroxidation.\textsuperscript{35}

No direct evidence is available concerning the basic mechanism contributing to leukocyte activation in hypertensives. A potential candidate is the blood platelet, which is known to be activated in SHR.\textsuperscript{36} A limited data set in the literature suggests that platelet numbers are not increased in SHR,\textsuperscript{37,38} and platelets are known to produce mediators that could interact with the circulating leukocytes (for review, see Reference 39). The activation of other mediator pathways, such as arachidonic acid products, complements, short chain peptides, cytokines, or depletion of endogenous deactivators, such as adenosine or albumin, must also be considered. All of these modalities are purely speculative at this point, since there is no data set to support any specific pathway in hypertension. The possibility exists that activation of neutrophilic leukocytes may in turn lead to platelet-leukocyte interactions\textsuperscript{40} that could stimulate intrinsic blood clotting mechanisms and exacerbate monocyte activation and microvascular obstruction. Obviously further investigation is necessary, in particular with respect to the involvement of catecholamine release, which is known to lead to release of margarine leukocytes\textsuperscript{41} and thus possibly to an elevation of the circulating leukocyte pool.

**Immune Deficiency in Spontaneously Hypertensive Rats**

Evidence has been advanced favoring thymus-derived lymphocyte dysfunction in SHR\textsuperscript{42–46} accompanied by elevated serum immunoglobulin G levels. This circumstance has been interpreted as the development of natural cytotoxic autoantibodies against T lymphocytes. The animals are also more susceptible to infections with viruses, predisposing them to further dysfunction of the cellular immune system.\textsuperscript{47} Implantation of thymus tissue into young and developing SHR serves to partially suppress the development of an elevated blood pressure.\textsuperscript{46,48} Chronic immunosuppressive treatment with cyclophosphamide or cyclosporin has been found to lead to a partial reduction in blood pressure in SHR.\textsuperscript{49,50} Interleukin treatment (by interleukin-2) was reported...
to restore blood pressure to normotensive levels in SHR after a single treatment at a young age, although this report failed to indicate the response in the normotensive WKY rat. Interleukin treatment was reported to be ineffective in Goldblatt hypertensive rats. Temporary reduction of blood pressure in young SHR during maturation has also been observed after Aspirin treatment.

Food restriction of young SHR during maturation has also been observed after Aspirin treatment. Temporary reduction of blood pressure in young SHR during maturation has also been observed after Aspirin treatment. Temporary reduction of blood pressure in young SHR during maturation has also been observed after Aspirin treatment. Temporary reduction of blood pressure in young SHR during maturation has also been observed after Aspirin treatment. Temporary reduction of blood pressure in young SHR during maturation has also been observed after Aspirin treatment.

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330 Hypertension Vol 17, No 3 March 1991


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