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Subchronic Toxicity of Aniline Hydrochloride in Rats

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Abstract. Hematological, biochemical and histopathological responses of subchronic exposure to aniline hydrochloride (AH) have been investigated in rats. Male Sprague-Dawley rats were given 600 ppm of AH in drinking water while the control rats received tap water only. Five rats from each group were sacrificed at 30, 60, and 90 days of treatment. Organ-to-body weight ratio for spleen in the AH-treated rats was 56, 61, and 53% higher than controls at days 30, 60, and 90, respectively. Liver showed a biphasic pattern for this ratio, a decrease at 30 days and then an increase at 60 days. Among other organs, testes showed a significant decrease in this ratio at 60 days. Hematological analysis showed 65% increase in WBC counts at 30 days in the AH-treated rats, whereas, no changes were recorded at later time points. Erythrocyte counts in the AH-treated rats showed very significant decreases at all the time points, whereas, hemoglobin and hematocrit decreased at 30 and 90 days of treatment. Mean corpuscular volume and mean corpuscular hemoglobin increased in the AH-treated rats at 60 and 90 days of treatment. Methemoglobin content showed significant increases of 89, 59 and 45% at days 30, 60, and 90, respectively. Among serum immunoglobulins, IgA in the AH-treated groups showed 24 and 51% increases at days 60 and 90, respectively. Analysis of splenic lymphocyte subpopulation showed a decrease in the T-helper ($CD4^+/CD8^-$) sub-set at 90 days whereas, other subpopulations were not affected. Aniline hydroxylase activity in the liver microsomes of the AH-treated rats was significantly higher at 60 days of treatment. At all times, spleen demonstrated striking histopathological changes including marked red pulp expansion due to increased sinusoidal cells, fibroblasts, and markedly increased light brown pigment of heme origin. Focal pericapsular fibrosis was found at all times, with no evidence of neoplasia. These histological changes were greatly accentuated with the progression of exposure. Our data, apart from indicating toxicity to the hemopoietic system, show a good interrelationship between damage to erythrocytes and splenic lesions associated with aniline exposure.

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Aniline, a toxic aromatic amine, is a widely used industrial chemical with an annual production of over 900 million lbs in the United States (Chem Eng News 1992). The main toxic effect of aniline, akin to several other aromatic amines, is the formation of methemoglobin (MetHb) which interferes with the oxygen-carrying capacity of the blood (Gralla *et al.* 1979; Beard and Noe 1981; Kim and Carlson 1986). The formation of MetHb could result from the oxidation of hemoglobin by aniline metabolites such as phenylhydroxylamine, 2-aminophenol and 4-aminophenol of which phenylhydroxylamine has been demonstrated to be the principal mediator of aniline-induced methemoglobinemia in rats (Harrison and Jollow 1986). *In vivo* exposure to aniline causes hemolysis and hemolytic anemia, toxicity to spleen and produces splenic tumors at higher doses (Bus and Popp 1987). Although a number of studies have documented involvement of spleen in the aniline induced toxicity (Gralla *et al.* 1979; Goodman *et al.* 1984; Weinberger *et al.* 1985), the molecular and cellular mechanisms by which aniline causes selective damage to spleen are not very well understood. Since a major function of the spleen is to remove aged or damaged erythrocytes, chemically-induced toxicity in these cells would result in their accumulation within the spleen. Therefore, to delineate the mechanisms of splenic toxicity of aniline, it is important to understand and follow the toxic responses in the blood chemistry profile which could contribute to the observed toxicity to the spleen. In this communication we have studied the interrelationship of blood changes, especially erythrocytes, with the splenic lesions as a function of time after subchronically exposing rats to aniline hydrochloride.

Materials and Methods

Animals and Treatment

Male Sprague-Dawley rats (~200 g), obtained from Harlan Sprague-Dawley Inc., Indianapolis, IN, were housed in wire bottom cages over absorbent paper with free access to tap water and Purina Lab chow. The animals were acclimated for 7 days in a room set for 12 h light/dark cycle. Our initial studies show that a rat weight ~200 g consumed ~20 ml of water each day. The solutions of aniline hydrochloride (AH), prepared in tap water, were adjusted to pH 6.8. The animals were divided into two groups of 15 rats each. One group received 600 ppm aqueous solution of AH, whereas, the other group received tap water

Table 1. Organ weight-to-body weight ratio ($\times 10^{-4}$) in rats treated with aniline hydrochloride[a]

Organs	30 Days	60 Days	90 Days
Heart	36.61 ± 1.97	33.20 ± 1.35	32.87 ± 3.61
	(36.85 ± 1.40)	(35.07 ± 1.32)	(30.36 ± 1.81)
Lung	46.50 ± 0.63	47.11 ± 4.06	42.86 ± 5.11
	(48.83 ± 3.56)	(45.10 ± 2.12)	(41.11 ± 2.93)
Liver	383.61 ± 12.70*	349.92 ± 16.21*	320.43 ± 25.22
	(412.19 ± 24.57)	(328.27 ± 8.97)	(322.68 ± 12.18)
Kidney	35.48 ± 3.07	32.60 ± 2.23	30.25 ± 2.46
	(37.07 ± 3.14)	(32.72 ± 1.62)	(30.81 ± 1.48)
Spleen	34.81 ± 2.69***	30.03 ± 5.90**	27.61 ± 3.02***
	(22.37 ± 2.86)	(18.66 ± 2.32)	(18.00 ± 1.72)
Testes	56.27 ± 6.50	42.83 ± 2.64*	43.00 ± 3.22
	(51.91 ± 4.19)	(47.03 ± 2.66)	(43.39 ± 2.19)
Brain	49.60 ± 2.06	44.11 ± 2.51	39.93 ± 2.44
	(51.49 ± 3.48)	(44.96 ± 3.88)	(40.00 ± 1.27)

[a] Values are mean ± S.D. of five rats
Control values are given in parentheses
*$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$ when compared to their respective controls

only and served as controls. Solutions were given in glass bottles and were changed on alternate days. Animals were weighed at weekly intervals for any body weight changes. The average consumption of aniline hydrochloride in this study was 60 mg/kg/day.

Experimental Procedures

Five rats from each group were sacrificed on days 30, 60, and 90 of the treatment. Animals were anesthetized with ether and the blood samples were withdrawn from the inferior vena cava. A small portion of the blood was quickly transferred to tubes containing EDTA for hematological analysis while the remaining blood was used to obtain serum. All major organs (liver, lung, heart, brain, kidney, spleen, thymus, testes and pancreas) were removed immediately, blotted and weighed. A portion of spleen was quickly transferred to a petri dish containing ice-cold phosphate-buffered saline (pH 7.2) and further processed for lymphocyte subpopulation analysis. Blood erythrocyte count, hemoglobin, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) content, hematocrit (HCT), platelets, white blood cell (WBC) count and the differential cell counts were determined on a Coulter Model ZB-1 blood cell counter (Coulter Electronics, Inc., Hialeah, FL) at the Hematology Laboratory, University of Texas Medical Branch.

A portion of each tissue was fixed in 10% buffered formalin and after standard histological processing the slides were stained with hematoxylin and eosin. The spleen sections were also stained with Pearl's Prussian blue for ferric iron deposition.

Biochemical and Immunological Assays in the Serum

Blood from control and AH-treated rats was stored overnight at 4°C and methemoglobin (MetHb) was determined according to the procedure of Betke et al. (1962). Serum lactate dehydrogenase (LDH) activ-

Table 2. Hematological changes in rats treated with aniline hydrochloride[a]

Parameters	30 Days	60 Days	90 Days
WBC ($\times 10^6$/ml)	13.69 ± 2.88*	9.88 ± 2.54	12.50 ± 1.95
	(8.31 ± 1.69)	(9.24 ± 1.80)	(11.93 ± 2.59)
RBC ($\times 10^9$/ml)	6.45 ± 0.62**	6.81 ± 0.24***	6.62 ± 0.25***
	(7.77 ± 0.20)	(7.80 ± 0.29)	(7.89 ± 0.27)
Hgb (g/dl)	12.34 ± 0.50***	13.28 ± 0.52	12.82 ± 0.29**
	(14.50 ± 0.43)	(13.82 ± 0.31)	(14.20 ± 0.65)
HCT (%)	37.22 ± 1.83*	40.08 ± 1.69	37.80 ± 0.75*
	(41.95 ± 1.80)	(40.20 ± 1.21)	(41.16 ± 1.98)
MCV (fl)	58.04 ± 5.45	58.82 ± 1.50***	57.16 ± 1.77***
	(53.90 ± 1.38)	(51.58 ± 1.14)	(52.16 ± 0.92)
MCH (pg)	19.30 ± 1.94	19.50 ± 0.41***	19.36 ± 0.55**
	(18.66 ± 1.94)	(17.75 ± 0.45)	(18.02 ± 0.31)
PLT ($\times 10^6$/ml)	897.2 ± 265.2	1027.0 ± 84.2	1003.2 ± 59.5
	(958.4 ± 39.3)	(1011.2 ± 122.3)	(908.8 ± 120.3)

[a] Values are mean ± S.D. of five animals
Control values are given in parentheses
*$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$ when compared to their respective controls

Table 3. Changes in blood methemoglobin (MetHb) content and liver microsomal aniline hydroxylase activity in rats treated with aniline hydrochloride[a]

Parameters	30 Days	60 Days	90 Days
MetHb (%)	17.87 ± 1.96***	14.48 ± 1.54**	17.69 ± 0.82***
	(9.45 ± 0.93)	(9.09 ± 1.60)	(12.19 ± 1.12)
Aniline hydroxylase	1.12 ± 0.34	1.09 ± 0.11*	1.24 ± 0.14
(nmol/mg protein/min)	(0.94 ± 0.19)	(0.83 ± 0.20)	(1.04 ± 0.23)

[a] Values are mean ± S.D. of five rats
Control values are given in parentheses
*$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$ when compared to their respective controls

Table 4. Changes in serum enzyme activities due to aniline hydrochloride exposure in rats[a]

Enzyme	30 Days	60 Days	90 Days
LDH	1022.7 ± 255.5	829.3 ± 462.0**	1336.0 ± 432.4
(units/ml)	(1474.7 ± 376.6)	(1862.6 ± 179.9)	(1269.0 ± 432.6
AST	100.2 ± 7.0*	80.0 ± 11.7*	124.8 ± 12.8
(SF units/ml)	(110.0 ± 4.6)	(107.2 ± 13.8)	(122.8 ± 9.2)
ALT	44.0 ± 1.8	32.8 ± 5.2	44.8 ± 5.0
(SF units/ml)	(47.7 ± 6.9)	(35.8 ± 4.9)	(42.0 ± 5.1)

[a] Values are mean ± S.D. of five animals
Control values are given in parentheses
*$P < 0.05$; **$P < 0.005$ when compared to their respective controls

Table 5. Serum immunoglobulin changes in rats treated with aniline hydrochloride[a]

Immunoglobulin (Ig)	30 Days	60 Days	90 Days
IgM (mg/L)	473.6 ± 33.2	557.4 ± 158.4	649.8 ± 161.3
	(569.2 ± 117.3)	(535.4 ± 85.3)	(593.2 ± 134.4)
IgG (mg/L)	26124.0 ± 3464.1	38000.0 ± 2725.8	44580.0 ± 4213.9
	(21180.0 ± 4491.8)	(30435.0 ± 7213.7)	(45000.0 ± 8394.7)
IgA (mg/L)	68.8 ± 11.0	67.5 ± 6.7*	73.8 ± 11.4*
	(59.1 ± 12.0)	(54.6 ± 9.9)	(48.7 ± 11.1)

[a] Values are mean ± S.D. of five animals in each group
Control values are given in parenthesis
*$P < 0.05$ when compared to their respective controls

ity was measured according to Wroblewski and LaDue (1955) while aspartate oxaloglutarate aminotransferase (AST/GOT) and alanine oxaloglutarate aminotransferase (ALT/GPT) were masured by using Sigma diagnostic kits. Serum immunoglobulins (IgM, IgG, IgA) were quantiated by using radial immunodiffusion kits (The Binding Site, Birmingham, England).

Hepatic Microsomal Aniline Hydroxylase Activity

Liver microsomes from control and AH-treated rats were prepared according to the method of deDuve *et al.* (1955). Aniline hydroxylase activity in the liver microsomes was assayed according to Ko *et al.* (1987).

Enumeration of Splenic Lymphocyte Subpopulation

Enumeration of splenic lymphocyte subpopulations was done according to the procedure described in our earlier paper (Khan *et al.* 1991).

Statistical Analysis of Data

Data were analyzed for statistical differences between experimental and control rats by using two-tailed Student's t-tests. Differences of $P \leq 0.05$ were considered to be significant.

Results

Organ Weight-to-Body Weight Ratio

Animals treated with AH did not show any statistically significant changes in the body weights when compared to controls at various time points. However, AH-treated rats did show organ weight changes and the ratio of organ weight-to-body weight in the control and AH-treated rats has been recorded in Table 1. Spleen showed the most significant increases in this ratio, being 56, 61, and 53% higher than controls at days 30, 60, and 90, respectively. Liver showed a biphasic pattern, a decrease of about 7% at 30 days and then an increase of about 7% at 60 days. Among other organs, testes showed a significant decrease of 9% at 60 days.

Table 6. Splenic lymphocyte subpopulations in aniline hydrochloride treated rats at 90 days[a]

Cell types	Cell population
T cells	106.06 ± 18.83
B cells	131.62 ± 34.10
T-helper	76.29 ± 4.62***
T-suppressor	105.62 ± 58.65

[a] Values are expressed as percent of controls ± S.D. of four animals
***$P < 0.0005$ as compared to control group

Hematological Changes Due to AH exposure

Table 2 shows the effect of AH treatment on various blood parameters. White blood cell (WBC) counts in the AH-treated rats increased by 56% at 30 days whereas, no changes were observed at 60 and 90 days. Differential WBC analysis did not show any significant change in any of the individual cell populations. RBC counts in the AH-treated rats decreased by 17, 13, and 16% at days 30, 60, and 90, respectively. Hemoglobin concentration and hematocrit values followed similar patterns, showing significant decreases at 30 and 90 days only. Among other parameters, the mean corpuscular volume and mean cor-

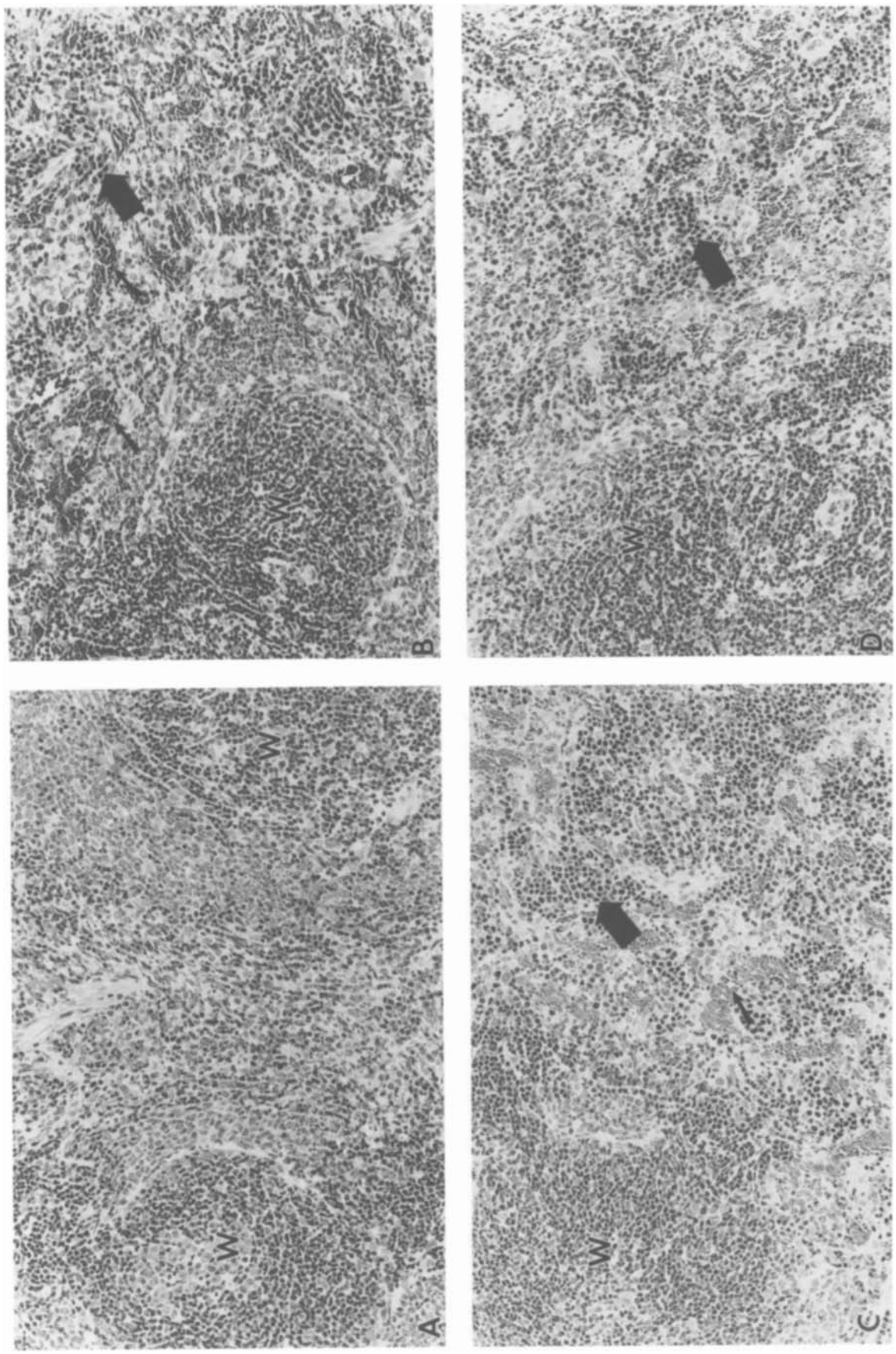


Fig. 1. Sections of spleen from rats given aniline hydrochloride; all sections × 160, hematoxylin and eosin. **A** Control spleen with two dark nodules of lymphoid tissue representing white pulp (W); the remainder of the photomicrograph represents red pulp. Each of the subsequent sections are similarly oriented with white pulp to the left only. **B** After 30 days of AH treatment, red pulp is expanded with marked congestion of blood vessels (small arrows) and aggregates of large macrophage-like cells (large arrow). **C** Congestion and aggregates of cells (large arrow) are increased after 60 days of AH treatment. **D** Progression of red pulp changes are pronounced after 90 days of AH treatment.

puscular hemoglobin showed significant increases in the AH-treated rats at 60 and 90 days of the treatment.

Changes in Methemoglobin Concentration

As evident from Table 3, MetHb content in the AH-treated rats showed very significant increases at all time points studied. The maximum increase was about 89% at 30 days followed by 59 and 45 at days 60 and 90, respectively. Since analysis of MetHb was performed 24 hr after the collection of blood samples, relatively higher values for the controls could be due to autoxidation of hemoglobin. However, the pattern of our results is in agreement with those reported by others (Jenkins *et al.* 1972; Kim and Carlson 1986).

Aniline Hydroxylase Activity in Liver Microsomes

Hepatic microsomal aniline hydroxylase activity in the AH-treated rats was found to be 20, 32, and 19% higher as compared to respective control values at days 30, 60, and 90, respectively (Table 3). However, these increases were statistically significant only at 60 days of treatment.

Changes in the Serum Enzyme Activities

Changes in the serum LDH, AST and ALT activities, which are markers of cell or tissue injury, are shown in Table 4. Interestingly, these enzyme activities showed decreases instead of increases at days 30 and 60. While LDH activity was only 45% of controls at 60 days, AST activity showed decreases both at 30 and 60 days, being 91% and 75% of controls, respectively. However, at 90 days, both LDH and AST activities were comparable to the control values. ALT activity did not show any significant change at any time point.

Changes in the Serum Immunoglobulins

Table 5 shows the alterations in the serum immunoglobulins as a result of AH exposure. IgA in the AH treated rats showed significant increases of 24 and 51% at days 60 and 90, respectively. IgG showed marginal though statistically insignificant increases of 23 and 25% at days 30 and 60, respectively. No changes were observed in the serum IgM levels at any stage of AH treatment.

Splenic Lymphocyte Subpopulations

Analysis of splenic lymphocyte subpopulation, as presented in Table 6, was done only at 90 days of exposure and showed a significant decrease in the T-helper ($CD4^+/CD8^-$) lymphocyte subpopulation at 90 days whereas, no changes were recorded in the T-cells, T-suppressor subset and B-lymphocyte populations.

Histopathologic Lesions

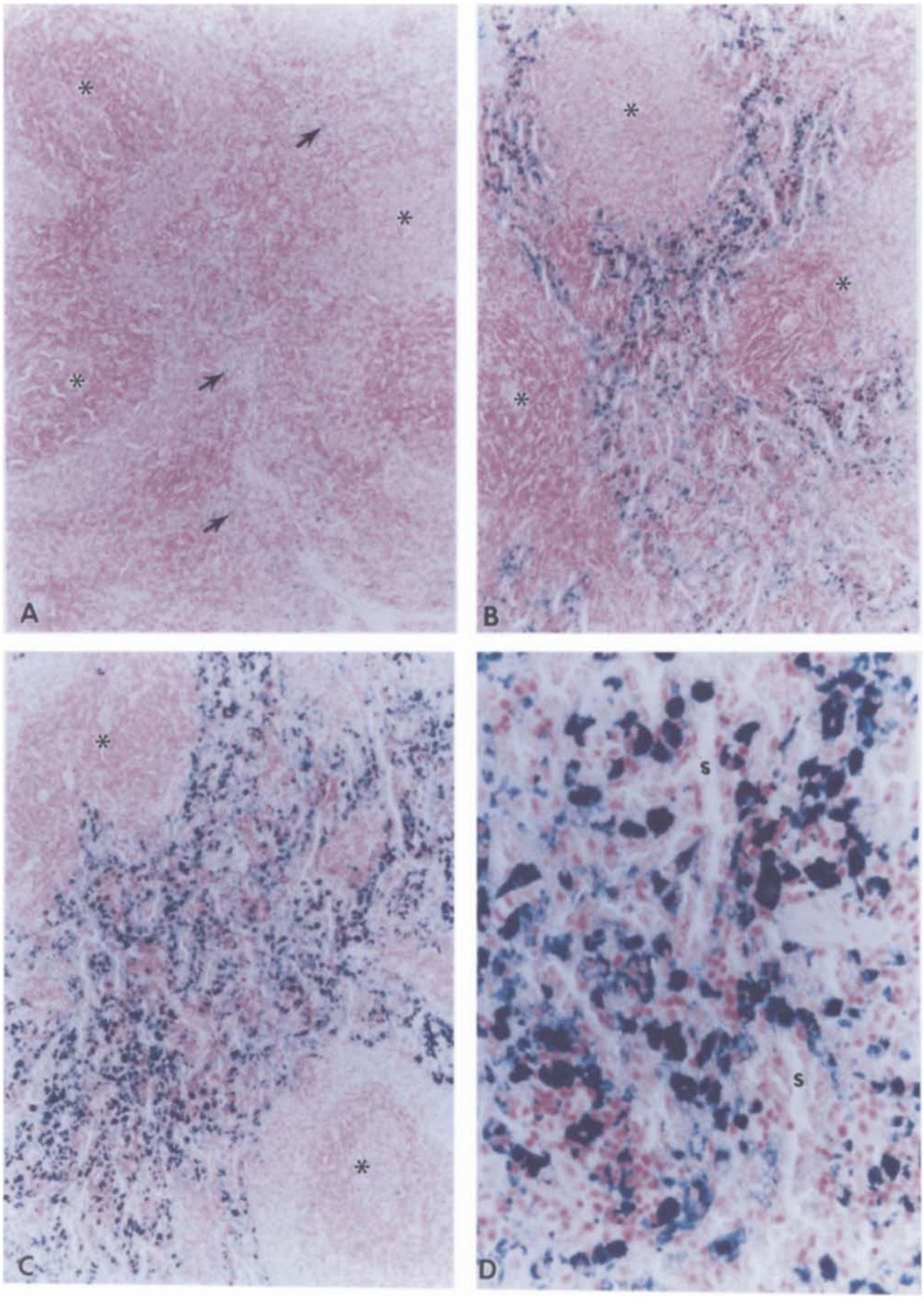
Histologic changes due to AH treatment were confined to the spleen only. As evident from Figure 1, at all time points spleen in the AH-treated rats demonstrated striking histopathology which included marked red pulp expansion due to increased splenic sinusoids, fibroblasts, and macrophages, and congestion of blood vessels. These changes appeared to be time dependent; at 90 days the red pulp changes were more pronounced than earlier time points studied. Focal pericapsular fibrosis was found at all times, but no evidence of neoplasia was seen. When the spleen sections from control and AH-treated rats were stained with Prussian blue for iron (Figure 2), the control spleen showed very scant, normal amount of iron, whereas spleen sections from rats given AH-treatment for 30 days showed marked accumulation of iron in the red pulp which was even more greatly accentuated at 90 days of the treatment. High power magnification of the red pulp from spleens of rats given AH treatment for 90 days demonstrated congested sinusoidal vessels and massive iron accumulation in macrophages (Figure 2).

Fig. 2. Histopathology of spleen; all sections are stained with Prussian blue for iron. **A** Control rat shows normal splenic architecture with white pulp areas (*) and red pulp between nodules of white pulp. Only very scant, normal amount of iron present (arrows); (×95). **B** Rat given AH for 30 days shows marked accumulation of dark staining iron in red pulp; (×95). **C** After 90 days on AH protocol, the iron accumulation in red pulp is greatly accentuated; (×95). **D** Higher power of red pulp from spleen of rat given AH for 90 days demonstrates congested sinusoidal vessels(s) and massive iron accumulation in macrophages; (×320).

Discussion

Several studies have documented the involvement of erythrocytes and spleen in aniline induced toxicity (Jenkins *et al.* 1972; Kim and Carlson 1986; Bus and Popp 1987). However, these studies were not designed to explore the relationship between the AH-related changes in the blood chemistry and splenic lesions, as well as the extent of their occurrence. The results of this investigation, apart from suggesting the erythrocytes and spleen as the targets of aniline toxicity, indicate that initial interaction of the compound with erythrocytes and their scavenging by spleen, could be the key factor in the development of splenic lesions, progression of which would depend upon the duration of exposure.

Aniline hydrochloride (600 ppm) was given to rats through drinking water for up to 90 days and changes were recorded in the blood, spleen and other tissues at days 30, 60, and 90 of the treatment. The data indicate AH-related decreases in the erythrocyte numbers, hemoglobin content and hematocrit values, but these decreases, though consistent, did not show any severity with the progression of exposure. Similarly, MetHb formation which is the dominant expression of toxic effect in erythrocytes in laboratory animals and humans as a result of exposure to a number of aromatic amino and nitro compounds (Beard and Noe 1981; Beutler 1985), also increased in the AH-treated rats at all time points, although the increases were less dramatic at the later time points. The mechanism(s) of formation and toxicological implications of MetHb by aromatic amino and nitro compounds have been studied extensively (Beard and Noe 1981). A recent study demonstrated that phenylhydroxylamine is the sole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mediator of aniline-induced methemoglobinemia in rats (Harrison and Jollow 1987). Thus, MetHb formation and the accompanying hemolytic anemia, as evident from decreases in erythrocytes and hemoglobin, are the early signs of aniline toxicity.

The gross enlargements of spleens at all three time points in the AH-fed rats are consistent with earlier findings of Gralla *et al.* (1979). Since a major function of the spleen is to remove aged or damaged erythrocytes, the increases in the spleen weights in AH-treated rats could be due to excessive deposition of damaged erythrocytes as a result of aniline toxicity and thus, splenomegaly would appear a secondary effect of erythrocyte toxicity. The spleens from AH-treated rats demonstrated striking and time-dependent histopathological changes including marked red pulp expansion due to increased sinusoids, fibroblasts and macrophages, and congestion of blood vessels. As expected, the progression of changes in red pulp were much more pronounced at day 90 than that at days 30 and 60. Also, marked accumulation of iron in the red pulp of AH-treated rats was evident and was greatly accentuated at later time points, especially at day 90 of AH treatment. Continuous exposure to aniline through drinking water could keep generating chemically-damaged erythrocytes, thus resulting in their accumulation in the spleen. The vascular congestion and marked iron deposition in the spleens, with the progression of exposure, may lead to splenic hemorrhage, formation of fibrous tissue mass, and again in conjunction with accumulation of aniline metabolites within the spleen (derived from scavenged erythrocytes), transformation of mesenchymal cells of the spleen. Deposition of erythrocyte debris, especially iron in the spleen, has been associated with tissue damage possibly mediated by iron-catalyzed free radical reaction (Heys and Dormandy 1981). Such reactions could result in a variety of tissue-damaging reactions, including lipid peroxidation, protein degradation and DNA strand breaks (Weir *et al.* 1984; Halliwell and Gutteridge 1986; Miller *et al.* 1992).

Aniline has been reported to stimulate the activities of the hepatic microsomal enzymes *in vitro* (Wisniewska-Knypl *et al.* 1975). Although the results of our study demonstrated the induction of aniline hydroxylase activity in the liver microsomes, the increments in the activities at various time points were relatively of low order to draw any meaningful conclusion regarding the role of this drug metabolizing enzyme in the metabolism and toxicity of aniline. Similarly, changes in the serum immunoglobulins, especially IgA and IgG, and decreases in splenic T-helper (CD_4^+/CD_8^-) population at 90 days were also observed as a result of AH treatment. Whether these changes reflect any immunotoxic potential of AH will need further studies, especially functional status of lymphocytes.

In conclusion, our study demonstrates that erythrocyte damage and splenic toxicity due to aniline exposure are closely associated processes and the progression of splenic lesions is dependent on the duration of the exposure. Further, this study demonstrates that iron content in the spleens of AH-treated rats increases substantially with the progression of the exposure apparently due to deposition of chemically-damaged erythrocytes. This overload of iron may result in the generation of iron-catalyzed free radicals which can cause oxidative damage to spleen. This possibility needs to be substantiated experimentally.

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