Protracted Anemia Associated with Chronic, Relapsing Systemic Inflammation Induced by Arthropathic Peptidoglycan-Polysaccharide Polymers in Rats

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Mild hypoproliferative anemia with abnormal iron metabolism frequently accompanies chronic inflammation and infection in humans. To determine whether anemia is associated with chronic relapsing arthritis induced by bacterial cell wall polymers, serial determinations of the hematocrit were measured in rats injected intraperitoneally with sonicated peptidoglycan-polysaccharide fragments from group A streptococci. Acute anemia peaked 5 to 10 days after injection, and chronic, spontaneously relapsing anemia persisted for 309 days. 51Cr labeling demonstrated decreased erythrocyte survival, i.e., a half-life of 8.4 days in cell wall-injected rats versus 11.8 days in controls. Erythrocytes were mildly microcytic, and leucocyte counts were elevated during early spontaneous reactivation of arthritis, 15 days after injection of peptidoglycan-polysaccharide. Bone marrow myeloid/erythroid precursor ratios were elevated in arthritic rats (P < 0.0001). Purified peptidoglycan produced an acute anemia lasting 10 days, while injection of group A streptococcal polysaccharide and mutanolysin-digested cell wall did not affect the hematocrit. The minimal effective dose of peptidoglycan-polysaccharide was 5 μg of rhamnose per g (body weight). Serum iron and transferrin levels were decreased in cell wall-injected rats (P < 0.005) and were closely correlated with hematocrit values and joint inflammatory scores. Stainable iron was increased in the liver, spleen, and mesenteric lymph nodes and unchanged in the bone marrow of cell wall-injected rats. Anemia accompanying chronic, relapsing systemic inflammation induced by peptidoglycan-polysaccharide polymers appears to be an excellent animal model of the anemia of chronic disease.

Mild nonprogressive anemia accompanies chronic inflammation of diverse etiologies, including chronic bacterial and fungal infections (42), rheumatoid arthritis (43), Crohn’s disease (10), and widespread malignancies (13). The anemia of chronic disease (inflammation) is characterized by mildly decreased circulating erythrocyte (RBC) survival, lack of an appropriate bone marrow compensatory response, and disordered iron kinetics (13, 24, 48). Typically, the serum iron, total iron-binding capacity (TIBC), and marrow sideroblasts are decreased in spite of normal-to-increased bone marrow and tissue iron stores. Recent studies have suggested that monokines secreted by activated macrophages may be responsible for suppression of bone marrow erythroid precursors (33, 37). Anemia accompanies several experimental inflammatory states associated with macrophage activation, including adjuvant arthritis (25), arthritis induced by viable BCG (26) and Mycoplasma arthritidis (11), and turpentine abscesses (32). These experimental models have been proposed as animal models of the anemia of chronic inflammation, however, none of the models produce protracted anemia nor exactly reproduce the bone marrow suppression and disordered iron metabolism found in the anemia of chronic disease (48).

Bacterial cell wall polymers induce well-characterized acute and chronic granulomatous inflammation in a variety of organs after local and systemic injection (35, 41). Peptidoglycan-polysaccharide (PG-APS) polymers derived from group A streptococci induce an acute and chronic systemic inflammatory response characterized by hepatic granulomas (20, 47) and spontaneously relapsing arthritis (8) that can persist at least 1 year after a single intraperitoneal (i.p.) injection into susceptible rat strains. Macrophages are activated to secrete increased amounts of interleukin-1 (IL-1) by both in vitro and in vivo exposure to PG-APS (L. A. Bristol and J. H. Schwab, submitted for publication).

This study is designed to determine whether a single i.p. injection of PG-APS would produce protracted anemia and to determine whether this well-characterized and reproducible chronic, systemic inflammatory response in genetically susceptible rats could serve as a reliable model of the anemia of chronic disease. We demonstrate that PG-APS produces a chronic, spontaneously relapsing anemia that persists at least 10 months after a single i.p. injection and whose course closely parallels activity of peripheral arthritis. PG is the moiety of PG-APS responsible for the anemia and arthritis. PG-APS-induced anemia resembles human anemia of chronic disease in the following respects: it is mild to moderate, RBC survival is decreased, RBCs are slightly microcytic, serum iron and transferrin concentrations are decreased, and reticuloendothelial iron stores are normal or increased.

(A portion of this work was presented as a poster at the 87th Annual Meeting of the American Society for Microbiology in Atlanta, Ga., 1 to 6 March 1987, and was published as an abstract [R. B. Sartor, S. K. Anderle, W. J. Cromartie, and J. H. Schwab, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, B-146, p. 49].)
MATERIALS AND METHODS

Animals. Female inbred Lewis rats were purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass., and were housed 2 or 3 per cage with free access to Purina rat chow and water at all times. Rats weighed 140 to 160 g at the time of injection.

Cell wall polymer preparation. PG-APS polymers were isolated under aseptic conditions from the cell walls of strain D58 of group A streptococci (Streptococcus pyogenes) as previously described in detail (40). Briefly, streptococci grown in Todd-Hewitt broth (BBL Microbiology Systems, Cockeysville, Md.) were disrupted in a Braun MSK cell homogenizer (Bronwell Scientific, Inc., Rochester, N.Y.) and the cell wall fraction was separated by filtration and centrifugation. The cell walls were extracted three times in 2% sodium dodecyl sulfate (Sigma Chemical Co., St. Louis, Mo.) at 56°C and then washed extensively and dialyzed to remove the sodium dodecyl sulfate. Purified PG-APS polymers (400 mg) were suspended in 20 ml of sterile phosphate-buffered saline (PBS) (pH 7.2) and sonicated for 35 min using a 0.5-in. (1.27-cm) probe and a setting of 8 (model 350; Branson Sonic Power Co., Danbury, Conn.). The suspension of the method of Chetty et al. (5). The M-1 fraction (pH 7.2) and sonicated for 3 min with a 9-kilocycle Sonic oscillator (Raytheon Co., Waltham, Mass.) to remove large particles. Purified PG-APS fragments prepared by this method have molecular weights ranging from 5 × 10^6 to 5 × 10^10 (16). Sterility was confirmed by culturing 0.1 ml on sheep blood agar. PG-APS concentrations for injections were calculated on the basis of rhamnose values determined by the method of Dische and Shettles (9). Immediately before injection, PG-APS was diluted to appropriate concentrations in PBS (pH 7.2) and sonicated for 3 min with a 9-kilocycle Sonic oscillator (Raytheon Co., Waltham, Mass.) to disperse aggregates.

Preparation of PG-APS fractions. Group-specific APS and PG were prepared by hot formamide treatment of cell walls. APS was purified from the supernatant by the method of Krause (22), as modified by Chetty et al. (4), and contained 47% rhamnose. PG was purified by multiple formamide extractions by the method of Krause and McCarty (23) and contained 0.5% rhamnose.

Mutanolysin-digested PG-APS was prepared by a modification of the method of Chetty et al. (5). The M-1 fraction of mutanolysin, an endo-N-acetylmuramidase derived from Streptomyces globisporus, was obtained from Miles Laboratories, Inc., Elkhart, Ind. Samples (8 ml) of sonicated PG-APS (27.4 mg of rhamnose) were diluted with 12 ml of PBS containing 0.5 M sodium citrate and 0.05 mM MgCl2, containing 3.29 mg of mutanolysin and then incubated at 37°C with constant mixing for 11 h. The optical density measured at 600 nm decreased from 0.492 to 0.135 after incubation with mutanolysin. Mutanolysin-digested PG-APS has been previously shown to have a molecular weight of <5 × 10^8 and to produce only transient edema without arthritis upon i.p. injection (5).

Labeling of erythrocytes with 51Cr. RBCs from Lewis rats were labeled in vitro with 51Cr as previously described (34). 51Cr-labeled RBCs were diluted to 1.07 × 10^6 cpm/ml in PBS and injected within 30 min.

Experimental design. Lewis rats were injected i.p. with 1 ml of PG-APS or cell wall fraction diluted with PBS (pH 7.2) to the appropriate concentration (rhamnose concentrations cited in individual experiments). In each experiment, control rats were injected i.p. with 1 ml of PBS. In the study measuring RBC survival, 0.5 ml of 51Cr-labeled RBCs (5.35 × 10^5 cpm) was injected intravenously 30 min before i.p. injection of PG-APS or PBS. Serial determinations of hematocrit (HCT), 51Cr counts per minute, or sedimentation rates were obtained at intervals outlined in individual experiments from blood derived from transection of the tip of the tail. A minimal volume of blood was obtained to prevent iron deficiency. Bleeding was controlled by digital pressure on the tail above the transection and by cautery immediately after blood collection. Blood was collected directly into two heparinized 75-mm-long microhematocrit capillary tubes (approximately 70 μl of operational volume; Fisher Scientific, Pittsburgh, Pa.) for duplicate HCT and sedimentation rate determinations and into heparinized microcentrifuge tubes (approximately 200-μl volume) for 51Cr or automated cell counting. Serum for iron and transferrin levels was obtained at necropsy by cardiac puncture. All injections and blood collections were done under ether inhalation anesthesia to minimize discomfort, and animals were killed by CO2 overdose.

Measurement of laboratory and clinical parameters. HCT values were determined in duplicate microhematocrit tubes immediately after centrifugation at 12,600 × g for 3 min. Spontaneous sedimentation of RBCs was measured in microhematocrit capillary tubes allowed to stand upright for 30 min after collection at room temperature. The percent plasma relative to total volume was read with a microhematocrit capillary tube reader (Lancer, Brunswick Co., St. Louis, Mo.). The amount of 51Cr within 0.1 ml of heparinized blood was determined in a gamma counter (1197 series; Automatic Gamma Counting System; Searle Analytic, Inc., Des Plains, Ill.). Counts per minute at each time point were corrected for the spontaneous decay in 51Cr activity (physical half-life, 27.7 days). An automated cell counter (150 series; Baker Instruments Corp., Allentown, Pa.), programmed for rat blood cells, was used for hemoglobin, RBC and leukocyte (WBC) counts, and mean corpuscular volume determinations. Transferrin was measured immunonutritiometrically (31) with the following modifications. Rat sera were diluted 1:25 with PBS. Rabbit antiserum to human transferrin antiserum (Nordic Immunological Reagents, El Toro, Calif.) was diluted 1:50 with 40 g of polyethylene glycol per liter of PBS. Within-run imprecision for the transferrin assay was less than 5%. The iron concentration was determined by using Beckman’s ferrozine method (Beckman Instruments, Inc., Brea, Calif.) by the instructions of the manufacturer. The analytical imprecision for the iron assay was less than 4%. The determinations of transferrin and iron were performed on the Cobas-BIO centrifugal analyzer (Roche Analytical Instruments Inc., Nutley, N.J.). Iron stains were performed on femoral bone marrow touch preparations and Formalin-fixed liver and spleen sections by the method of Gomori (18). Bone marrow myeloid/erythroid precursor ratios were performed on touch preparations of femoral marrows, which were stained with Wright’s-Giemsa reagents. Bone marrow cellularity was determined in Formalin-fixed decalcified femurs, sternums, and ankle joints stained with hematoxylin and eosin. Arthritis was measured by using a clinical joint score of 0 to 4+ for each ankle (maximum score, 16) (8). We have previously demonstrated a close correlation of clinical joint scores with radiographic (6) and histologic (8) assessments of joint inflammation.

Statistical evaluation. Mean values of each experimental parameter were calculated for each group of rats and compared by Student’s unpaired t test. Experimental parameters were correlated by linear regression analysis.
ANEMIA INDUCED BY BACTERIAL CELL WALLS

RESULTS

Time course of anemia and arthritis. Serial HCTs were measured in female Lewis rats injected with PG-APS (20 µg of rhamnose per g) or PBS. PG-APS-injected rats developed a transient increase in HCT 17 h after injection, followed by a protracted anemia compared with the PBS-injected control group (Fig. 1). The time course of anemia followed a typical pattern in this and subsequent experiments. An acute drop in HCT occurred by 3 days after injection, with the lowest HCT values (36.2% in the PG-APS group versus 45.1% in the control group [P < 0.0001]) recorded 10 days after injection. Over the next week, the HCT slowly increased but never reached control values. Between 3 and 5 weeks after injection, individual rats developed a spontaneous recurrence of persistent anemia. The peak of the chronic anemia at this dose of PG-APS occurred 49 days after injection (HCT value of 36.4% ± 2.8% versus 46.5% ± 1.1% in the control group, [P < 0.0001]). HCT values in the PG-APS-injected rats were significantly lower than those of PBS-injected controls for 309 days after injection. Arthritis, as measured by the clinical joint score, demonstrated a similar acute phase, which peaked 3 days after PG-APS injection, and a chronic, spontaneously relapsing phase beginning 4 weeks after injection (Fig. 1). Mean values obscure the occasional dramatic change in the HCT and joint scores of individual rats during the spontaneous relapses and remissions of anemia and arthritis. The sporadic nature of the recurrence of anemia led to relatively high standard deviations of HCT values 3 to 4 weeks after injection (Fig. 1). All rats injected with PG-APS abruptly developed recurrent anemia (HCT of ≤40% and a decrease in HCT by >3 percentage points within 1 week); 57% (4 of 7) had a reactivation of arthritis within 1 week of the sudden drop in HCT. Recurrent anemia preceded or accompanied spontaneous reactivation of arthritis, which in the Lewis rat persists indefinitely as an anklylosed joint with massive bony proliferation (20). Control rats injected with PBS developed neither anemia nor arthritis.

RBC survival. Survival of syngeneic RBCs labeled in vitro with 51Cr was compared in rats injected with PG-APS (20 µg of rhamnose per g [body weight]) or PBS. PG-APS-injected rats had significantly lower counts per minute per 100 µl of blood than the controls did, beginning 7 days after injection.
TABLE 1. Ability of PG-APS constituents to induce anemia

<table>
<thead>
<tr>
<th>Material injected</th>
<th>HCT (%) (mean ± SEM) on day:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>PG-APS*</td>
<td>45.6 ± 0.5*</td>
</tr>
<tr>
<td>PG-APS digest†</td>
<td>43.7 ± 0.4</td>
</tr>
<tr>
<td>PG‡</td>
<td>41.1 ± 0.6*</td>
</tr>
<tr>
<td>APS*:</td>
<td>44.2 ± 0.6</td>
</tr>
<tr>
<td>PBS§</td>
<td>43.5 ± 0.4</td>
</tr>
</tbody>
</table>

* for P < 0.01 compared with the PBS-injected group; **, P < 0.001 compared with the PBS-injected group.
† PG-APS polymers from group A streptococci (30 µg of rhamnose; approximately 90 µg [dry weight] of PG-APS per g [body weight]) injected i.p. into six female Lewis rats.
‡ PG-APS polymers from group A streptococci (30 µg of rhamnose per g [body weight]) predigested for 12 h with mutanolysin and then injected i.p. into six rats.
§ Purified PG (45 µg [dry weight]/g [body weight]) injected i.p. into six rats.
∥ Formamide-extracted group A streptococcal APS (30 µg of rhamnose per g [body weight]) injected i.p. into six rats.
∥∥ PBS injected i.p. into six rats (buffer control).

and extending until 51Cr was no longer detectable (Table 2). The calculated half-life of blood cells, based on four half-lives, was 8.4 days in the PG-APS-injected rats compared with 11.8 days in controls. There was no evidence of gastrointestinal blood loss by Hemoccult (Smith-Kline Diagnostics, Sunnyvale, Calif.) testing of fecal samples and no evidence of bleeding from the cauterized tails. These results demonstrate a moderate decrease in RBC survival in PG-APS-treated rats which was not due to bleeding.

Active moiety of PG-APS. To determine which portion(s) of the PG-APS polymer was responsible for protracted anemia, we measured HCTs of rats injected with equivalent high doses of purified PG and APS as well as enzymatically degraded PG-APS (Table 1). As described above, rats injected with nondigested PG-APS polymers developed acute and chronic anemia and arthritis after a transient increase in HCT 1 day after injection. Rats injected with PG had arthritis and significantly decreased HCTs from 1 to 10 days after injection, with peak anemia at 6 days, but no evidence of chronic anemia or arthritis. In contrast, rats injected with APS or mutanolysin-digested PG-APS did not demonstrate decreased HCT values or arthritis at any time point but did develop transient peripheral edema 30 min after injection as previously described (4, 5). PG-injected rats did not develop edema, but four of six rats had clinically detectable diarrhea, and all six had conjunctivitis.

Dose response. Rats were injected with PG-APS in doses ranging from 0 to 40 µg of rhamnose per g (body weight) to determine the minimally effective dose that produced acute and chronic, relapsing anemia (Table 2). Rats injected with 0.5 or 1 µg of rhamnose per g (body weight) did not develop clinically significant anemia at any time, although sporadic time points showed a small but statistically significant difference in HCT compared with control values. PG-APS in doses between 10 and 40 µg of rhamnose per g (body weight) induced acute anemia peaking at 4 to 7 days after injection in a dose-dependent fashion. Rats injected with 5 to 40 µg of rhamnose developed chronic, spontaneously relapsing anemia with very interesting differences in the timing of the recurrences, depending on the dose injected (Table 3). Rats receiving 5 µg/g (body weight) i.p. had no acute change in HCT but developed chronic anemia 35 to 113 days after injection. Only one rat in this group had spontaneous reactivation of anemia. Rats receiving 10 to 20 µg of rhamnose per g (body weight) developed recurrent, chronic anemia which, in most animals, never returned to normal levels. These rats developed a recurrence of moderate (HCT of <40%) anemia 18 to 28 days after PG-APS injection; the second and third recurrences of anemia occurred 50 to 113 days after injection. In contrast, HCTs of rats receiving the highest dose of PG-APS (40 µg of rhamnose per g [body weight]) returned to completely normal levels by 10 days after injection and remained no different than control levels until 70 days after injection (Tables 2 and 3). Eventually, five of nine rats injected with 40 µg of rhamnose PG-APS per g (body weight) developed moderate chronic anemia, but none had a second relapse during the 135-day observation period.

Iron and transferrin levels. To determine whether iron metabolism was abnormal during chronic inflammation induced by PG-APS, serum iron and transferrin levels were

TABLE 2. Anemia with increasing doses of PG-APS polymers

<table>
<thead>
<tr>
<th>PG-APS dose ‡</th>
<th>HCT (%) (mean ± SEM) on day:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7</td>
</tr>
<tr>
<td>0</td>
<td>44.3 ± 0.3</td>
</tr>
<tr>
<td>0.5</td>
<td>43.2 ± 0.4</td>
</tr>
<tr>
<td>1</td>
<td>43.6 ± 0.5</td>
</tr>
<tr>
<td>5</td>
<td>44.4 ± 0.3</td>
</tr>
<tr>
<td>10</td>
<td>42.9 ± 0.4*</td>
</tr>
<tr>
<td>15</td>
<td>40.9 ± 0.5**</td>
</tr>
<tr>
<td>20</td>
<td>41.9 ± 0.2*</td>
</tr>
<tr>
<td>40</td>
<td>39.6 ± 0.8**</td>
</tr>
</tbody>
</table>

* PG-APS polymers from group A streptococci injected i.p. into six to nine female Lewis rats per group. Values are in micrograms of rhamnose per gram (body weight).
‡, P < 0.02 compared with rats given no PG-APS; ‡‡, P < 0.001 compared with rats given no PG-APS.
measured 36 days after i.p. injection of PG-APS (15 μg of rhamnose per g [body weight]). PG-APS-injected rats had significantly lower iron and transferrin levels than buffer-injected control rats (Table 4), despite one misinjected rat in the PG-APS group which did not develop arthritis, anemia, or abnormal iron and transferrin levels.

Bone marrows of rats injected with PG-APS 15 days prior to sacrifice contained normal concentrations of intracellular iron by cytochemical staining. By 33 and 65 days after injection, iron staining within the bone marrow was diminished compared with iron staining in controls but remained detectable. Iron-containing macrophages were markedly increased in livers of rats injected with PG-APS, with intensely staining enlarged macrophages concentrated at the periphery of hepatic granulomas (Fig. 3), portal tracts, and central veins. Kupffer cells of PG-APS-injected rats frequently contained detectable iron and were randomly distributed. In contrast, livers of control rats had rare Kupffer cells containing faintly staining iron, which were usually adjacent to central veins. Mesenteric lymph nodes and spleens of PG-APS-injected rats contained increased numbers of iron-containing macrophages, frequently present in aggregates and giant cells.

**Correlation of experimental parameters.** PG-APS-injected rats which developed anemia consistently displayed concurrent arthritis, increased RBC sedimentation rates, hepatic granulomas, and decreased serum iron and transferrin levels (Table 4). There was a striking temporal relationship between arthritis and delayed onset or recurrent anemia (Fig. 4) as well as between the acute phase of inflammation and anemia (Fig. 1). In pooled experiments, 35 of 39 (90%) rats which developed recurrent anemia had their first reactivation of arthritis within 1 week of the abrupt drop in HCT. At four time points between 10 and 36 days after injection of PG-APS or PBS, HCT and joint scores were very closely correlated, with $r$ values ranging from $-0.93$ to $-0.98$ and $P < 0.0001$. Owing to variability in the timing of spontaneously reactivating inflammation within an experimental group, mean values did not adequately demonstrate the close correlation that existed between HCT values and joint scores during recurrence of arthritis and anemia in individual rats (Fig. 4). Lewis rats develop irreversible destructive arthritis

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**TABLE 3. Incidence and timing of severe anemia (HCT of <40%) with increasing doses of PG-APS**

<table>
<thead>
<tr>
<th>PG-APS dose</th>
<th>Acute anemia</th>
<th>Chronic anemia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Peak</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>5/8</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>5/9</td>
</tr>
<tr>
<td>15</td>
<td>4/7</td>
<td>7/7</td>
</tr>
<tr>
<td>20</td>
<td>5/9</td>
<td>7-10</td>
</tr>
<tr>
<td>40</td>
<td>5/7</td>
<td>4</td>
</tr>
</tbody>
</table>

* See footnote a of Table 2.

**TABLE 4. Correlation of experimental parameters in cell wall-injected and control rats**

<table>
<thead>
<tr>
<th>Material injected</th>
<th>Mean ± SEM</th>
<th>HCT (%)</th>
<th>Serum iron (μg/dl)</th>
<th>Serum transferrin (mg/dl)</th>
<th>RBC sedimentation (%)</th>
<th>Joint score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>38.8 ± 2.7*</td>
<td>149 ± 49*</td>
<td>30.4 ± 12.9*</td>
<td>3.8 ± 1.3**</td>
<td>6.4 ± 1.6*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>47.7 ± 0.2</td>
<td>358 ± 19</td>
<td>79.0 ± 1.5</td>
<td>0.2 ± 0.1</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Correlation coefficient</th>
<th>HCT</th>
<th>Joint score</th>
<th>Transferrin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.97</td>
<td>0.98</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>0.97</td>
<td>0.97</td>
<td>0.95</td>
</tr>
</tbody>
</table>

* *, $P < 0.005$ compared with PBS-injected rats; **, $P = 0.01$ compared with PBS-injected rats.

PG-APS polymers (15 μg of rhamnose per g [body weight]) injected i.p. into five rats 36 days before sacrifice.

PBS injected i.p. into five rats 36 days before sacrifice.
FIG. 4. Temporal relationship between delayed onset of anemia and arthritis in a female Lewis rat injected i.p. with PG-APS polymers (5 μg of rhamnose per g). This rat did not develop anemia or arthritis until 42 and 50 days, respectively, after injection of a low dose of PG-APS. The anemia was maximal at the onset of arthritis.

with bony proliferation and ankylosis, leading to persistently elevated joint scores because of ankle enlargement due to new bone formation, but no gross evidence of active inflammation was detected by edema and erythema. Anemia was better correlated with active joint inflammation than relatively inactive chronic joint destruction (Fig. 1) and usually immediately preceded reactivation of arthritis in individual rats (Fig. 4). Similarly, increased spontaneous sedimentation of RBCs was associated with recurrence of anemia and arthritis (Table 5) and returned to normal values during remission of active inflammation.

Rats studied during the first spontaneous reactivation of arthritis, 15 days after PG-APS injection, developed severe anemia which was accompanied by significantly decreased hemoglobin and RBC counts (P ≤ 0.001, Table 6). The mean corpuscular volume of the PG-APS-injected rats was slightly lower than that for control rats. PG-APS-injected rats also had elevated WBC counts, which were closely correlated with HCT values (r = 0.93) and joint scores (r = 0.94). The bone marrows of these rats had decreased numbers of RBC precursors relative to WBC precursors, as demonstrated by their significantly elevated bone marrow myeloid precursors/erythroid precursor ratios (Table 6). Bone marrow histology revealed normal-to-increased cellularity, with increased numbers of megakaryocytes and granulocyte precursors, in the PG-APS-injected rats compared with PBS-injected controls.

**TABLE 5.** HCTs, sedimentation rates, and joint scores during reactivation of PG-APS-induced inflammation

<table>
<thead>
<tr>
<th>Rat no. or mean</th>
<th>PG-APS-injected rats</th>
<th>PBS-injected rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCT (%)</td>
<td>Sedimentation rate</td>
</tr>
<tr>
<td>1</td>
<td>36</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>47.5</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>28.5</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>31.5</td>
<td>24</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>36</td>
</tr>
</tbody>
</table>

Mean ± SEM

34.7 ± 3.4
c
17.1 ± 6.1
d
5.5 ± 1.5
c

a Values measured 21 days after i.p. injection of PG-APS (15 μg of rhamnose per g [body weight]).

b Spontaneous sedimentation of RBC measured as percentage of total capillary tube volume 30 min after collection.

c P < 0.003 compared with PBS-injected rats.

d P = 0.02 compared with PBS-injected rats.

**DISCUSSION**

Anemia associated with acute and chronic systemic inflammation induced by PG-APS has several unique features which make it a useful model to study the anemia of chronic inflammation. PG-APS-induced anemia was protracted, as it persisted for at least 10 months after a single aqueous i.p. injection, and spontaneously relapsed and remitted, with its clinical course correlating with exacerbations and remissions of arthritis. Anemia following bacterial lipopolysaccharide (endotoxin) injection is transient and very mild (17). Formalin-killed *Corynebacterium parvum* injected into mice (27), infection with *M. arthritidis* (11) or BCG (26), and footpad injection of complete Freund adjuvant (25) induce anemia which is monophasic, with no evidence of spontaneous recurrence, and is limited to at most 8-weeks duration (26). PG-APS is resistant to biodegradation (39) and persists within macrophages for at least 4 months after injection (8, 35). Spontaneous reactivation of inflammation following a single local or systemic injection of PG-APS is a characteristic feature of inflammation induced by these phlogistic complexes and may be due to slow intracellular degradation of polymers with episodic release from tissue stores (14).

In the anemia of chronic disease, serum iron, transferrin, and TIBC are low despite normal or increased bone marrow and tissue iron stores (13, 24, 48). Rats injected with PG-APS had significantly lower serum iron and transferrin concentrations in the chronic phase of inflammation compared with those of control rats, and there was a strong correlation between iron and transferrin levels and the activity of arthritis. Macrophages within the liver, spleen, and mesenteric lymph nodes had increased stainable iron, particularly within giant cells and granulomas. Fibrotic, more-advanced hepatic granulomas had more densely staining iron than recently formed, active granulomas with necrotic centers, suggesting increased sequestration of iron with chronic inflammation. Mice injected with Freund adjuvant have slightly decreased plasma iron, but decreased TIBC and transferrin saturation develop only late in the course of anemia (25) and splenic iron is diminished, rather than increased, after repeated injections of adjuvant (28). The anemia secondary to BCG infection is not associated with hypoferremia (26). Injection of *C. parvum* into rats leads to a prompt decrease in serum iron and retention of iron within mononuclear phagocytes but an increased plasma TIBC (3). Similarly, mice with turpentine-induced inflammation (2) or infected with *M. arthritidis* (11) have a prompt decrease in plasma iron but an increase in transferrin and TIBC. Thus, PG-APS-induced chronic anemia in rats more closely mimics...
the clinical abnormalities of iron metabolism found in the anemia of chronic disease than previously described models of chronic inflammation or infection.

The demonstration that the PG fraction induced anemia is consistent with previous observations that polymeric PG is the active moiety responsible for most inflammatory reactions induced by PG-APS fragments from a variety of bacterial species (20, 41). Digestion of PG by the muramidase, mutanolysin, abrogates acute and chronic arthritis (20) and prevents acute vascular permeability changes in the gut-associated lymphoid tissue induced by PG-APS (R. B. Sartor, unpublished data). Purified PG is readily degraded by lysozyme, in contrast to PG covalently bound to APS (1). This observation is consistent with the transient anemia and arthritis which resolved by 14 days after injection of isolated PG with no evidence of recurrence, while PG-APS produced protracted, spontaneously relapsing anemia and arthritis. Purified group A streptococcal APS produced transient peripheral edema, as previously described (4), but no anemia or chronic arthritis.

The mechanism of anemia induced by PG-APS is unknown but probably involves both decreased RBC survival and synthesis. The rapid decrease in HCT during the acute phase and spontaneous recurrence of anemia, combined with the slightly decreased RBC half-life measured by $^{51}$Cr labeling, indicates that RBC survival is diminished. There was no evidence of gastrointestinal or external loss of RBCs, and the purified PG-APS preparation used for these studies did not contain streptococcal hemolysin. Patients with rheumatoid arthritis have a 20% decrease in RBC survival (43), and rats with adjuvant arthritis have a circulating $^{51}$Cr-RBC half-life of 10.2 days compared with 13.4 days in controls (28). One mechanism of shortened RBC survival is phagocytosis of RBCs by reticuloendothelial cells, which occurs in C. parvum-treated mice (27). Splenic macrophages normally clear senescent RBCs from the circulation. Macrophages in the spleen, liver, bone marrow, and lymph nodes can phagocytose RBCs that have subtle membrane damage or are coated with antibody or complement (7). Activation of macrophages is a common feature of many types of inflammation and has been postulated to be important in the pathogenesis of the anemia of chronic disease (24, 33, 37). PG-APS activates macrophages in vitro and in vivo (39; Bristol and Schwab, submitted), and intravenous injection of PG-APS leads to phagocytosis of RBCs by macrophages within mesenteric lymph nodes (34). PG activates complement (12), and Przywansky et al. (30) have demonstrated in vitro aggregation of RBCs and phagocytic cells via PG-APS-complement (C3b) bridging. Erythropagocytosis by activated reticuloendothelial cells could explain increased stainable iron within macrophages of the liver, spleen, and mesenteric lymph nodes of PG-APS-injected rats with anemia. Another possible explanation of increased macrophage iron is sequestration of lactoferrin-iron complexes after liberation of lactoferrin from the secondary granules of PG-APS-activated polymorphonuclear leukocytes.

The primary defect in the anemia of chronic disease appears to be the lack of an appropriate bone marrow response to the slight decrease in RBC survival (13, 48). Figure 2 shows only a slightly increased destruction of RBCs during the relatively abrupt recurrence of anemia 3 to 5 weeks after PG-APS injection, indicating variable suppression of RBC synthesis during different phases of experimental inflammation. Activated macrophages have been shown to suppress erythropoiesis both during stimulation with LPS (36, 37) or in disseminated histoplasmosis infection (50). Mouse peritoneal macrophages activated by human recombinant tumor necrosis factor or Cryptococcus neoformans infection suppress erythroid progenitor cell growth (33). Lee (24) speculated that IL-1 mediates the anemia of chronic disease, but it is apparent that IL-1 has no direct erythroid inhibitory effect (21, 33, 37), although this monokine activates polymorphonuclear leukocytes to release lactoferrin, causes a decrease in serum iron (24), and is produced by scattered bone marrow cells (44). IL-1 has indirect regulatory effects on hematopoiesis by stimulating bone marrow stromal cells (15), T lymphocytes (19), fibroblasts (21), and endothelial cells (38) to release multilineage hematopoietic growth factors and activating fibroblasts to release soluble factors that regulate early hematopoietic progenitor cells (51). Thus, IL-1 may be responsible for the leukocytosis observed after PG-APS injection. Recently, Tracey et al. (46) demonstrated that twice-daily administration of tumor necrosis factor to rats for 8 days induced a progressive anemia which appeared within 3 days and was associated with a leukocytosis. We postulate that the chronic anemia induced by PG-APS is a result of peptidoglycan activation of macrophages which liberate monokines, including tumor necrosis factor (45) and IL-1 (Bristol and Schwab, submitted), and soluble inflammatory mediators, such as prostaglandin E$_2$ (49), which may suppress erythropoiesis directly or indirectly. Alternatively, stimulated T cells, which have been implicated in the pathogenesis of PG-APS-induced inflammation (47), may suppress the bone marrow, as has been demonstrated in rheumatoid arthritis (43) and BCG infection in mice (29).

PG-APS-induced inflammation in rats provides an excellent model for examining protracted, spontaneously relapsing anemia with modestly decreased RBC survival and altered iron metabolism associated with well-characterized arthritis. PG-APS is chemically and antigenically defined and can activate macrophages in vivo or in vitro, permitting more thorough evaluation of mechanisms of bone marrow suppression. Activation of macrophages by PG-PS may be a common mechanism of the anemia accompanying chronic infection by many bacterial species.
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LITERATURE CITED


ANEMIA INDUCED BY BACTERIAL CELL WALLS


