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Bacterial Cell Wall Polymers (Peptidoglycan-Polysaccharide) Cause Reactivation of Arthritis

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Intraperitoneal (i.p.) injection of peptidoglycan-polysaccharide derived from group A streptococci (PG-APS) causes chronic arthritis with spontaneous remissions and exacerbations. We hypothesized that, following i.p. injection, PG-APS released from hepatic stores mediated spontaneous recurrences of arthritis. We tested whether transplanted livers with large amounts of PG-APS were able to reactivate quiescent arthritis. Saline-loaded (group 1) or PG-APS-loaded (group 2) livers were transplanted into rats which had been injected intra-articularly 10 days earlier with PG-APS in one joint and saline in the other. A comparison was made with the arthritis that occurred in rats injected i.p. with PG-APS which did not receive transplants (group 3). Arthritis was monitored by serial measurement of joint diameters. Transplantation of saline-loaded livers (group 1) caused no reactivation of arthritis. However, transplantation of PG-APS-loaded livers (group 2) reactivated arthritis ($P < 0.0001$). Injection of PG-APS i.p. (group 3) induced the most-severe arthritis. PG-APS levels in plasma decreased with time, and PG-APS accumulated in the spleen in groups 2 and 3. Plasma and hepatic levels of PG-APS in rats injected i.p. with PG-APS were greater than levels in rats transplanted with PG-APS-loaded livers, which in turn were greater than levels in rats with saline-loaded livers. Plasma tumor necrosis factor did not correlate with recurrence of arthritis. Transplantation with PG-APS-loaded livers induced reactivation of arthritis in preinjured joints. The extent of arthritis was proportional to hepatic PG-APS content. Reactivation of arthritis may be mediated by slow release of liver-sequestered PG-APS or cytokines (not tumor necrosis factor) released by the liver.

Systemic injections of poorly biodegradable bacterial cell wall polymers induce chronic, spontaneously relapsing multiorgan inflammation in genetically susceptible rats. For example, a single intraperitoneal (i.p.) injection of peptidoglycan-polysaccharide derived from group A streptococci (PG-APS) induces chronic erosive polyarthritis which undergoes spontaneous remissions and exacerbations in inbred Lewis rats (2). This inflammation is biphasic, consisting of acute nondeforming arthritis, anemia, and mild leukocytosis and then spontaneous development of erosive arthritis, granulomatous hepatitis, splenic granulomas and necrosis, leukocytosis, and anemia with features of the anemia of chronic disease (18). These chronic features persist for at least 10 months, during which time two to five spontaneous reactivations of inflammation occur (18). The phenomenon of spontaneous reactivation is important because it is a unique feature of PG-APS-induced inflammation as compared with other agents which cause acute inflammation alone. The mechanism of spontaneous reactivation of inflammation is unknown, and these experiments begin to explore this area.

Nearly all bacteria contain peptidoglycan-polysaccharide (PG-PS) in their cell walls, and although these polymers are very similar, there are subtle differences between the PG-PS from each different bacterial species. Several factors contribute to the chronic, spontaneously relapsing inflammation. (i) Poorly biodegradable PG-PS complexes which persist within inflamed tissues and the reticuloendothelial system cause chronic inflammation, whereas rapidly degradable PG-PS induces only transient inflammation (3, 23). The persistence of PG-PS within tissues correlates with the resistance of these polymers to lytic enzymes. (ii) Inbred rats demonstrate different genetic susceptibilities to bacterial cell wall polymers since Lewis rats develop a biphasic response but Buffalo and Fischer rats develop only transient inflammation (32). (iii) The dose of PG-PS injected affects the onset and chronicity of inflammation (6, 18). (iv) Since cyclosporin A and athymic rats (31) and monoclonal antibody against rat T-cell receptor (33) prevent chronic inflammation induced by PG-PS, the chronic phase of PG-PS-induced inflammation is under the control of activated T lymphocytes. (v) The size of the PG-PS polymers is also important since bacterial cell wall polymers of smaller molecular size (less than 5×10^6 Da) induce transient acute inflammation, but larger-molecular-size polymers (5×10^6 to 500×10^6 Da) cause chronic relapsing inflammation (6).

A unique and unexplained feature of chronic arthritis after systemic injection of PG-APS is that spontaneous reactivation occurs during the chronic phase of inflammation. Janusz et al. and Stimpson and coworkers postulated that PG-PS sequestered within the reticuloendothelial system (mainly phagocytic cells in the liver, spleen, bone marrow, and lymph nodes) is slowly degraded and then released to reactivate a previously injured joint (9, 27). After i.p. PG-APS injection, there is rapid uptake by reticuloendothelial

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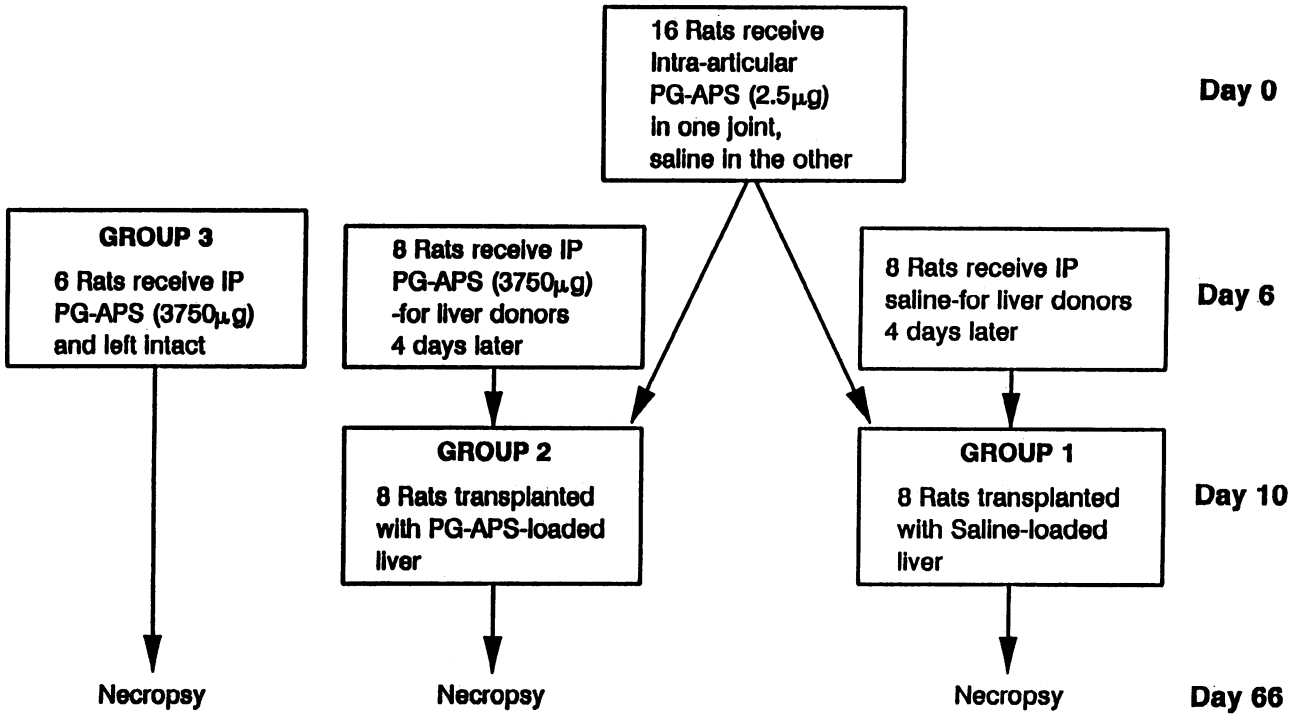

FIG. 1. Experimental protocol. This figure illustrates the experimental design and describes the groups of rats studied. Group 1 consists of eight rats which received PG-APS in one ankle joint and saline in the other ankle joint 10 days prior to receiving a liver transplantation. The donor liver was from a rat which had been injected with saline i.p. 4 days prior to transplantation. Group 2 consists of eight rats which had received PG-APS in the one ankle joint and saline in the other ankle joint 10 days prior to liver transplantation. The donor was from a rat which received PG-APS i.p. 4 days prior to transplantation. Group 3 consists of six rats which received PG-APS i.p. and were left intact until necropsy. Group 4 consists of 12 rats not injected with PG-APS either i.p. or intra-articularly and not transplanted, i.e., normal rats. All doses of PG-APS are expressed in rhamnose weight, which is approximately 30% of the PG-APS dry weight.

cells. The liver sequesters 10% of PG-APS 4 days after i.p. injection (4, 8), mostly localized to Kupffer cells (3), and this is the greatest amount of PG-APS found in a single organ. Forty-five days after i.p. injection, liver tissue concentrations decreased to approximately 1% of the initial injected dose, presumably by release into draining blood or lymphatics or by degradation by endogenous muralytic enzymes such as lysozyme (8, 27). It is postulated that PG-APS released from sequestered sites such as Kupffer cells redistributes to other tissues including the joint and may account for periodic reactivation of arthritis. Stimpson et al. demonstrated that PG-APS polymers in the blood and joints were smaller than the polymers found in the liver (27) and that liver extracts were able to degrade PG-APS (28). These findings indicate that the reticuloendothelial system releases partially degraded PG-APS polymers (28). Furthermore, local or systemic injection of PG-APS polymers can reactivate arthritis in a previously injured joint (5).

Thus, considerable indirect evidence suggests that slow release of partially degraded PG-PS by the liver and other reticuloendothelial organs may reactivate experimental arthritis. In the present study, we directly test this hypothesis by determining whether transplanted rat livers, loaded with sequestered PG-APS, are able to reactivate arthritis.

In these studies, we used a model in which reactivation of arthritis is sensitive to circulating PG-PS polymers (5). A single intra-articular injection of PG-APS into the tibio-talar (ankle) joint of a Lewis rat causes a self-limited, monoarticular arthritis that becomes clinically quiescent 10 to 21 days after intra-articular injection. Reactivation of arthritis can be achieved by systemic injections of PG-PS (5), purified peptidoglycan (5), endotoxin (26), or superantigen (21), intra-articular injection of platelet-derived growth factor, tumor necrosis factor alpha (TNF-α) (24, 29), or interleukin-1 (20, 25), and creation of small-bowel bacterial overgrowth (11). The intravenous dose of PG-APS necessary to reactivate arthritis in this model is very small, only 50 to 100 μg of rhamnose, which is 1 to 3% of the i.p. injected dose required to initiate chronic relapsing arthritis (20). In the present study, we found that transplantation of livers that had accumulated PG-APS caused reactivation of arthritis, suggesting that hepatic release of PG-APS polymers or cytokines can reactivate joint inflammation in this model.

MATERIALS AND METHODS

Experimental design. On day 0, 16 male, specific-pathogen-free Lewis rats (Charles River, Raleigh, N.C.) weighing 250 g were injected intra-articularly with 10 μl of PG-APS in a dose of 2.5 μg of rhamnose into one ankle joint, and 10 μl of pyrogen-free saline was injected into the contralateral ankle joint, as described previously (5). The PG-APS-injected joint was called the preinjured joint; the saline-injected joint was called the control joint. On day 10, when arthritis in the PG-APS-injected ankle was resolving, liver transplantation was performed (Fig. 1). Eight intra-articularly injected rats received a donor liver from rats which had received an i.p. injection of an arthropathogenic dose of PG-APS (PG-APS-loaded liver, group 2), and the other eight intra-articularly injected rats received a donor liver from rats

which had been injected i.p. with pyrogen-free saline (saline-loaded liver, group 1). Donor male Lewis rats were injected i.p. with PG-APS (3,750 μg of rhamnose; 15 μg/g of body weight) or i.p. with saline 4 days prior to removal of the liver for transplantation. PG-APS-injected rats developed acute arthritis prior to harvest of the liver for transplantation, indicating a systemic effect of PG-APS. Additionally, six male Lewis rats were injected i.p. with PG-APS (15 μg of rhamnose per g of body weight) but were left intact and not used as donors (group 3). These rats were killed 60 days after i.p. injection of PG-APS to measure the PG-APS contents in livers and spleens for comparison with those of rats which received transplanted livers. Tissues from 12 rats which had not been injected with PG-APS and which had received no liver transplantation were assayed for background levels of PG-APS (group 4).

Joint diameters were measured daily in duplicate by a blinded observer with a micrometer (25, 30) from day 0 to 21 and then weekly thereafter. Rats were weighed weekly. Plasma was drawn from the tail vein on day 0, every other day between days 10 to 21, and then weekly thereafter for determination of PG-APS, aspartate aminotransferase, and TNF-α levels.

When the experiment was completed 56 days after liver transplantation, the rats were killed by CO_2 inhalation, and the livers, spleens, and joints were harvested for histology and measurement of PG-APS levels by enzyme-linked immunosorbent assay (ELISA). As summarized in Fig. 1, the four groups of rats studied included (i) 8 rats with preinjured ankle joints which received saline-loaded livers by transplantation (group 1), (ii) 8 rats with preinjured ankle joints which received PG-APS-loaded livers by transplantation (group 2), (iii) 6 rats with no prior joint injury which received i.p. PG-APS and were left intact (group 3), and (iv) 12 rats which received no PG-APS and no liver transplantation (group 4).

Transplantation of livers. Rat livers were transplanted under ether anesthesia as described by Steffen et al. (22) and Kamada and Calne (10). To avoid rejection and graft-versus-host disease, syngeneic male Lewis rats (250 g) were used as recipients and donors. Donor livers were flushed via the portal vein with chilled University of Wisconsin solution (Madison, Wis.), cuffs were attached, and the explants were stored in University of Wisconsin solution at 4°C. Before implantation, the donor grafts were infused with 30 ml of Carolina rinse solution at 28 to 30°C (1). Recipient surgery including hepatectomy and transplantation required approximately 60 min. During this time, the portal vein was clamped for 15 min and the inferior vena cava was clamped for not more than 20 min. Rats were given food and water ad libitum postoperatively.

Measurement of TNF-α. Plasma samples were collected in aprotinin (0.67 trypsin inhibitor unit (TIU)/ml) and EDTA (1.5 mg/ml) as described previously (13), centrifuged, and stored at −70°C until assayed. A murine TNF-α ELISA kit (Genzyme, Boston, Mass.) was used (13). Ninety-six-well microtiter plates were coated with hamster anti-TNF-α antiserum in 100-μl volumes and incubated at 4°C overnight. After the plates were washed with phosphate-buffered saline–0.1% Tween 80 buffer, blocker was added for 2 h at room temperature. After the plates were washed, samples were diluted at least 1 to 4, applied in 100-μl volumes, and allowed to incubate for 18 h at 4°C. The plates were washed, donkey anti-TNF-α antiserum was added for 3 h at room temperature, and the plates were washed again. The goat anti-donkey peroxidase conjugate was then applied for another 3 h. The plates were washed, the substrate solution was applied, and color changes were monitored with an ELISA reader (Dynatech, Chantilly, Va.) at an optical density of 450 nm after 5 min. The assay measures murine TNF-α in the range of 50 to 1,000 pg/ml, which were concentrations used to construct a standard curve.

Preparation of PG-APS. The culture and harvesting of group A streptococci, isolation of the cell walls, and separation of the PG-APS fragments have been described in detail (27, 30). The amount of PG-APS in each preparation was determined by measurement of rhamnose (30), which constitutes approximately one-third of the dry weight of PG-APS. All values of PG-APS stated in these studies are expressed in rhamnose weight.

Measurement of PG-APS. Livers and spleens were homogenized in a tissue grinder in Tris buffer (pH 7.4) with 0.1% Tween 80 and 1% human serum albumin in a ratio of 4 ml of buffer to 1 g of tissue. Joints were frozen in liquid nitrogen, pulverized in a steel chamber with a hammer, diluted in Tris–0.1% Tween 80–1.0% human serum albumin buffer, and then sonicated (Ultrasonics, Farmingdale, N.Y.) for 60 s (4).

PG-APS was measured by ELISA as described previously (12). Briefly, 96-well microtiter plates were coated for 18 h at 4°C with affinity-purified rabbit anti-*N*-acetylglucosamine antibody. After the plates were washed with PBS–0.1% Tween 80 buffer, 0.1% bovine serum albumin was applied to prevent nonspecific binding. Diluted samples were then added and incubated for 18 h at 4°C. After the plates were washed, biotinylated affinity-purified rabbit anti-*N*-acetylglucosamine antibody was added for 3 h at room temperature. Plates were again washed, and avidin conjugated with alkaline phosphatase was added for another 3 h. The color reaction was developed by using *p*-nitrophenyl phosphate substrate (substrate tablets; Sigma Chemical Co., St. Louis, Mo.), and the A_{405} was measured 30 min later with a Dynatech ELISA reader. The amount of PG-APS was calculated from a standard curve for purified PG-APS based on rhamnose content.

Measurement of anti-PG-PS antibodies. Anti-PG-APS antibodies were measured in joint tissues which were frozen and pulverized as described above. Anti-PG-APS immunoglobulin G (IgG) and IgM were measured by an ELISA as described previously (12). Briefly, 96-well microtiter plates were coated with 100 μl of a 2-μg/ml concentration of PG-APS prepared as described above. After 18 h of incubation at 4°C, the plates were washed and blocked with 0.1% bovine serum albumin for 2 h at 25°C. Samples of joint tissue were diluted at least fourfold and applied to the plates for 18 h at 4°C. Alkaline phosphatase-conjugated goat anti-rat IgG or IgM (Sigma) was added for 8 h at 4°C, and then color was developed with the Sigma substrate tablets as described above. The optical density was measured at 405 nm after 90 min for this assay.

Histological techniques. When rats were killed, specimens of liver were fixed in 10% buffered formalin and then processed for histological examination and stained with hematoxylin and eosin. For the determination of number and location of granulomas and the number of nonparenchymal cells, slides were coded and read blindly by a single observer (S.N.L.).

Statistics. Weights, plasma aspartate aminotransferase, hematocrits, and PG-APS and TNF-α levels were compared by using the two-tailed Student's *t* test. Joint diameters were compared by using repeated-measure analysis of variance. Differences were considered statistically significant when P was <0.05.

TABLE 1. Clinical and laboratory data for rats after liver transplantation

Expt group	n	No. of deaths	Aspartate aminotransferase (U/liter)[a]		Wt gain (g)[a]	Hematocrit (%)[a]
			Day 14	Day 56		
1[b]	8	0	87 ± 13	94 ± 15	77 ± 13	43 ± 2.8
2[c]	7	0	79 ± 19	74 ± 19	78 ± 31	43 ± 1.6

[a] All values are means ± standard deviations at the time of necropsy (56 days after transplantation) unless otherwise noted.
[b] Rats killed 56 days after being transplanted with livers from rats injected i.p. with saline.
[c] Rats killed 56 days after being transplanted with livers from rats injected i.p. with PG-APS (15 μg of rhamnose per body weight), 4 days before transplantation.

RESULTS

Surgical outcome. No rats in either group which had liver transplantation died. The arterial anastomosis was unsuccessful in one rat, which survived but developed an increased bilirubin level of 11.4 mg/dl. Despite surviving until the end of the experiment, the rat showed poor weight gain, a low hematocrit, and massive bile duct proliferation on histologic examination of the liver. This rat had received a PG-APS-loaded liver transplantation and was excluded from the study. The other rats which received grafts all grew similarly, with initial weight loss for 10 to 14 days and then gradual weight gain until they were killed. Weight gains, hematocrits, and plasma aspartate aminotransferase values at 2 and 8 weeks were no different between rats with saline-loaded livers and those with PG-APS-loaded liver transplantations (Table 1).

Joint swelling. The mean changes in the preinjured joint diameters of groups 1, 2, and 3 are shown in Fig. 2 and 3A. In rats injected i.p. with PG-APS (group 3), both joints developed arthritis, and the right ankle joint was not significantly more swollen than the left ankle joint. The left ankle was arbitrarily chosen to be compared with the preinjured joints of groups 1 and 2. Preinjured ankle joints, which were injected with PG-APS, showed reactivation of arthritis following transplantation with PG-APS-loaded livers (Fig. 2 and 3A). Both the absolute joint diameters (data not shown) and the change in joint diameters were significantly greater in rats that received PG-APS-loaded livers by transplantation than in those that received saline-loaded livers. An example of the gross swelling of a joint during an episode of reactivation of arthritis is shown in Fig. 4. The peak joint diameters occurred 42 days after transplantation of PG-APS-loaded livers and after i.p. injection of PG-APS. Rats which received PG-APS i.p. (group 3) showed the greatest increase in joint diameters for both ankles, neither of which had been preinjured intra-articularly with PG-APS.

In Fig. 3, the changes in joint diameter of each rat are illustrated. An increase in joint diameter is considered significant when it is greater than 0.30 mm. Five of seven rats which received PG-APS-loaded livers (group 2) had an increase in the preinjured joint diameter of more than 0.30 mm compared with two of eight rats with increased joint diameters in the saline-loaded liver controls (group 1). By using repeated-measure analysis of variance, we found highly significant differences among groups 1, 2, and 3 ($P <$

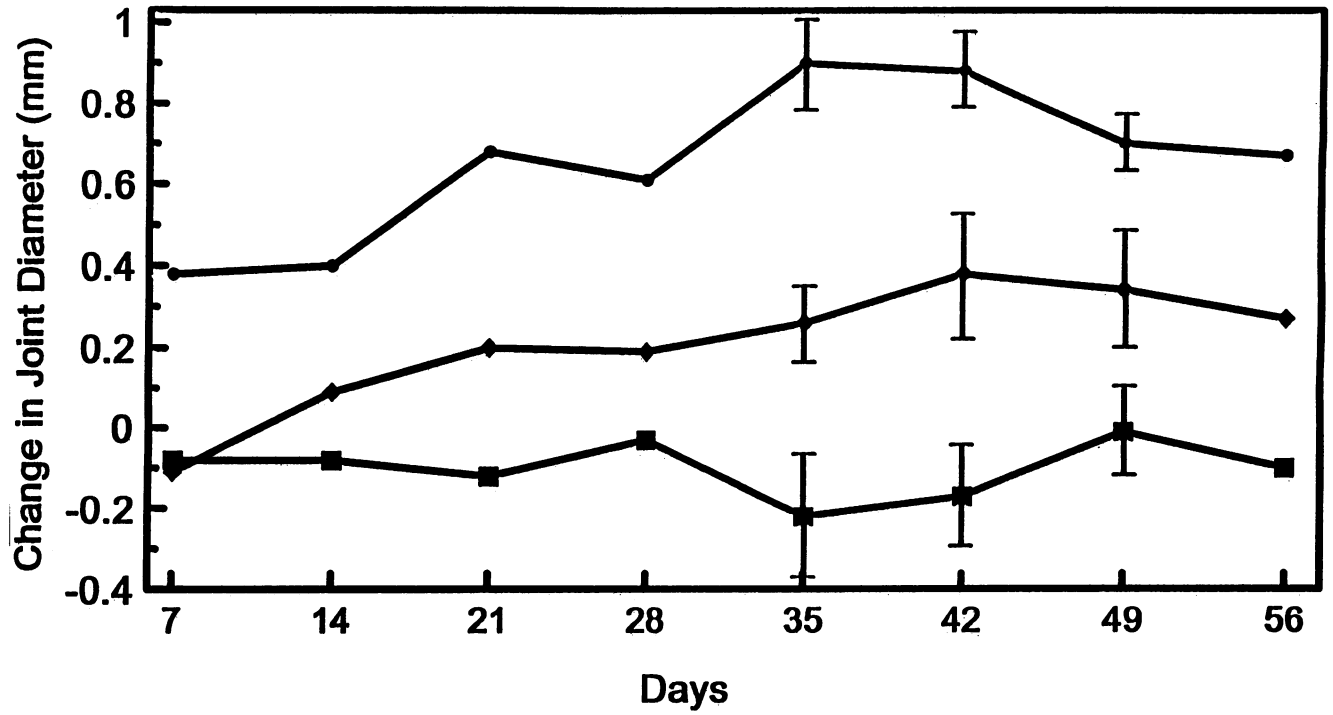

FIG. 2. Change in joint diameters after i.p. injection of PG-APS (group 3; ●), transplantation with PG-APS-loaded livers (group 2; ◆), and transplantation with saline-loaded livers (group 1; ■). Groups 1 and 2 had been preinjured with an intra-articular injection of PG-APS 10 days prior to surgery. Values represent means ± standard errors. The horizontal axis refers to time in days after i.p. injection of PG-APS or liver transplantation surgery. On days 35, 42, and 49, the changes in joint diameter were significantly greater in groups 2 and 3 than in group 1 (rats which received saline-loaded livers), as determined by the Student's t test ($P < 0.05$).

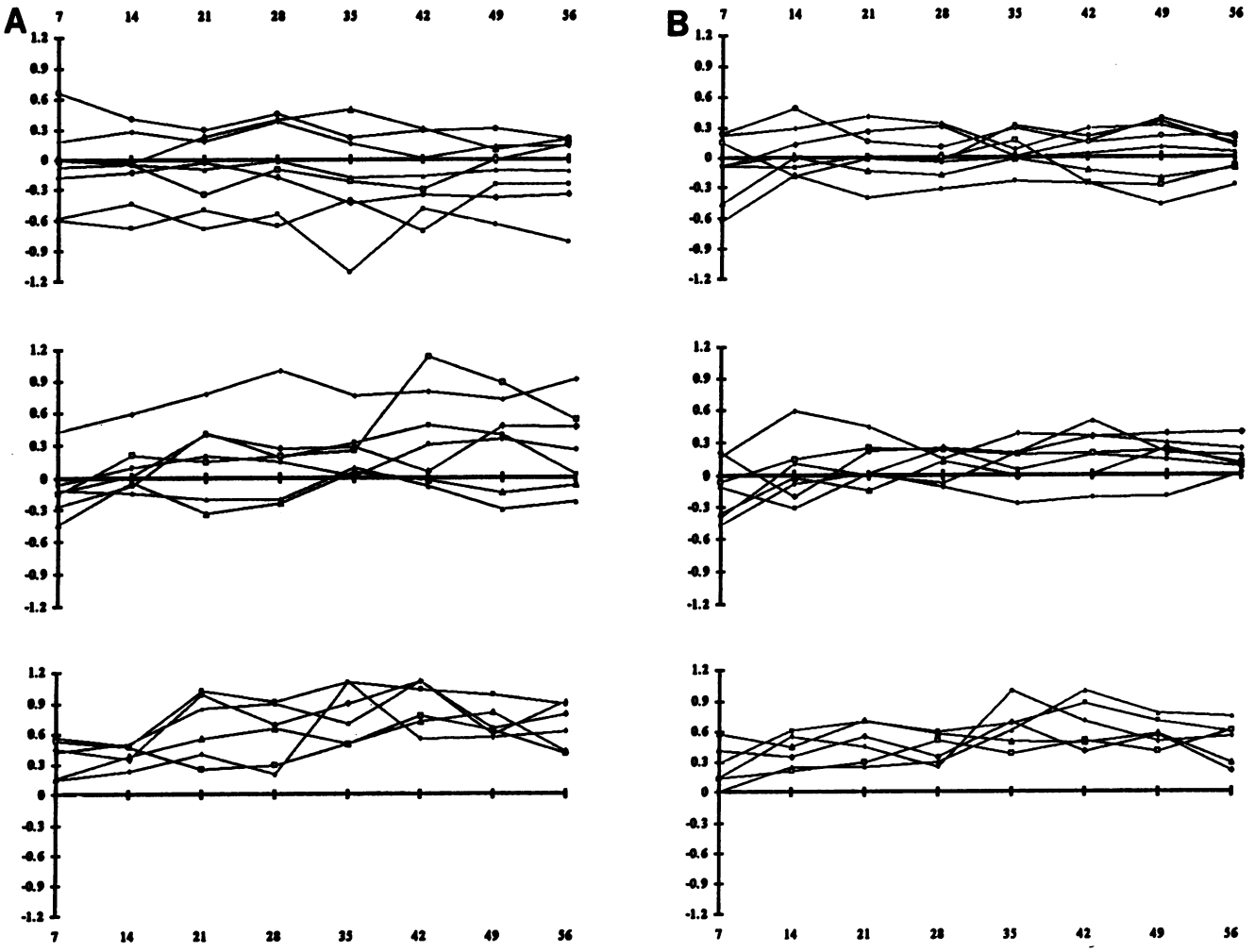

FIG. 3. Individual change in joint diameters for both ankle joints of each rat following i.p. injection of PG-APS and liver transplantation. The horizontal axis represents time in days after i.p. injection of PG-APS or liver transplantation. (A) Changes in joint diameter in preinjured (PG-APS-injected) ankle joints of groups 1 and 2 and the arbitrarily chosen left joint of group 3. (Top) Changes in joint diameter of rats which received intra-articular PG-APS and then a saline-loaded liver transplantation 10 days later (group 1). These joints showed no significant increase in diameter after transplantation. (Middle) Results of rats which received intra-articular PG-APS and then a PG-APS-loaded liver transplantation 10 days later (group 2). (Bottom) Change in joint diameter after i.p. injection of PG-APS (group 3). By using repeated-measure analysis of variance, the three groups demonstrated significant differences ($P < 0.0001$). (B) Changes in Control (saline-injected) ankle joints of rats in groups 1 (top) and 2 (middle) and the right ankle of group 3 (bottom). Only joints from rats injected i.p. with PG-APS (group 3) demonstrated statistically significant increases in diameter. However, saline-injected joints in three of seven rats which received PG-APS-loaded livers (group 2) showed an increased joint diameter greater than or equal to 0.3 mm, which indicates arthritis.

0.0001). Control ankle joints, injected with pyrogen-free saline, showed no significant change in diameter regardless of the source of the transplanted liver (Fig. 3B).

Plasma and tissue PG-APS levels. When rats were killed 56 days after transplantation, hepatic PG-APS levels were markedly greater in those animals which received PG-APS-loaded livers than those which received saline-loaded livers ($P < 0.001$; Table 2). Transplanted PG-APS-loaded livers had 17-fold less PG-APS than livers of rats which received PG-APS i.p. but were left intact (group 3). Saline-loaded livers contained amounts of PG-APS similar to those in livers from rats never injected intra-articularly or i.p. with PG-APS (normal rats). To create group 2, the rats were initially injected i.p. with PG-APS (3,750 μg of rhamnose), the livers were removed after 4 days and transplanted to a new host, and the rats were then killed 56 days later. At this time, the livers contained 1.74 μg of rhamnose, which is 0.05% of the initial i.p. injected dose of PG-APS. Rats in group 3 also were initially injected with PG-APS (3,750 μg of rhamnose) i.p. but were left intact. Thirty micrograms of rhamnose was recovered from the livers of rats from group 3 (i.e., 0.80% of the original i.p. dose of PG-APS), which was significantly greater than the amount recovered from transplanted PG-APS-loaded livers (Table 2; $P < 0.001$).

PG-APS concentrations in spleens of rats receiving PG-APS i.p. but left intact (group 3) were 15-fold higher than the concentrations in spleens of rats which had received PG-APS-loaded livers by transplantation (group 2). However, splenic PG-APS levels in group 2 were 33-fold greater than levels in spleens of rats transplanted with saline-loaded livers (group 1), indicating accumulation of PG-APS from the transplanted liver.

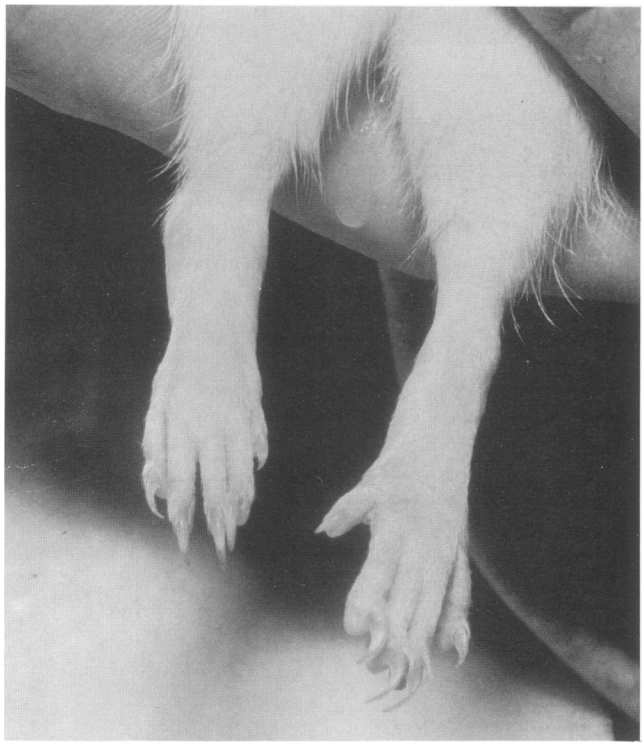

FIG. 4. Photograph of rat ankle joints. The preinjured right ankle joint had been injected at the beginning of the experiment with PG-APS (2.5 μg of rhamnose) in a 10-μl volume; the left ankle joint had been injected with 10 μl of saline. Forty-two days after transplantation with a liver which had been PG-APS loaded, the right ankle joint showed increased diameter, swelling, redness, and decreased range of motion compared with the left ankle joint.

By 56 days, differences in the amounts of PG-APS in joint tissue between the groups were not statistically significant, as measured by ELISA (Table 2). Joints injected with PG-APS did not have significantly higher PG-APS concentrations in tissue than the saline-injected contralateral joints 56 days after transplantation. Similarly, joints injected intra-articularly with PG-APS showed no significant differences in PG-APS content whether the rat received a saline-loaded or a PG-APS-loaded liver. Since anti-PG-APS antibody in joint tissue could potentially block PG-APS measurement by ELISA, anti-PG-APS IgG and IgM were measured in joint tissues, but no differences were found in antibody levels among the different groups of rats or between PG-APS-injected and saline-injected joints (data not shown). The addition of a known amount of PG-APS to joint tissue samples was 80 to 110% recovered, as measured by ELISA, indicating that no inhibitor to the detection of PG-APS by ELISA was present in joint tissue. Therefore, by using an ELISA at the end of the experiment, we could demonstrate no significant differences in PG-APS content between the ankle joints.

In group 2, the highest plasma level of PG-APS occurred at the first measurement, 2 days posttransplantation, and then progressively declined (Table 2). Considering a blood volume of 8% of total body weight, the amount of circulating PG-APS in the blood of a 250-g rat at 2 days posttransplantation (the time point with the highest measurement) would be 500 ng. Plasma PG-APS levels were not increased by intra-articular injection of PG-APS. At each time point until day 42, plasma PG-APS levels were consistently higher in rats which received PG-APS i.p. (group 3) than in those which were transplanted with a PG-APS-loaded liver (group 2), which, in turn, were higher than plasma PG-APS levels of rats with saline-loaded livers.

Plasma TNF-α levels. Plasma TNF-α levels in rats with saline-loaded livers did not change with time, were only slightly higher than levels in normal rats, and were at the lower end of detection by an ELISA. However, plasma from rats with PG-APS-loaded livers had elevated TNF-α levels measured between days 35 and 49. The peak plasma TNF-α level 42 days after transplantation with a PG-APS-loaded liver (121 ± 42 pg/ml [mean ± standard deviation]) was greater than the plasma TNF-α level in rats transplanted with a saline-loaded liver (48 ± 11 pg/ml; $P < 0.05$). Compared with controls, no significant increase in plasma TNF-α levels were detected in rats following i.p. injection of PG-APS (group 3) despite greater joint swelling at all time points.

Liver inflammation. When rats were killed 56 days after transplantation, there were no visible surface granulomas in the transplanted livers that had been loaded with PG-APS (group 2). The lack of surface granulomas is in contrast to

TABLE 2. Tissue and plasma PG-APS levels after liver transplantation

Expt group	n	PG-APS levels in:									
		Liver (ng/liver)[a]	Spleen (ng/spleen)[a]	Joints (ng/g)[a]	Plasma (ng/ml) on day:						
					2	14	28	35	42	49	56
1	8	35 ± 8	1.8 ± 1.8	11 ± 6[b] 8 ± 6[c]	0.3 ± 0.4	2.4 ± 1.5	2.0 ± 1.3	1.5 ± 0.9	1.4 ± 1.0	1.7 ± 1.1	0.9 ± 0.7
2	7	1,740 ± 340[d]	59 ± 8[d]	13 ± 7[b] 9 ± 7[c]	25 ± 8[d]	1.5 ± 3[d]	5.0 ± 1.9[d]	3.3 ± 1.6[e]	1.5 ± 0.3	1.2 ± 0.5	1.3 ± 0.5
3[f]	6	30,000 ± 4,500[d,g]	860 ± 112[d,g]	24 ± 18	177 ± 47[g]	93 ± 22[g]	21 ± 5[g]	9.1 ± 4.0[g]	3.3 ± 1.2[g]	1.8 ± 0.7	1.7 ± 0.7
4[h]	12	28 ± 12	2.1 ± 1.0	9 ± 5							

[a] Mean ± standard deviation of PG-APS in tissue 56 days after liver transplantation.
[b] Value for joints which had been injected intra-articularly with PG-APS.
[c] Value for joints which had been injected with pyrogen-free saline.
[d] Compared with rats with saline-loaded livers, $P < 0.001$.
[e] Compared with rats with saline-loaded livers, $P < 0.05$.
[f] Rats injected i.p. with PG-APS (15 μg of rhamnose per g of body weight) but not used as donors.
[g] Compared with rats with PG-APS-loaded livers, $P < 0.005$.
[h] Rats which have never been injected with PG-APS.

TABLE 3. Histology of livers from normal rats compared with those of livers 56 days after transplantation and after injection of PG-APS

Expt group	No. of animals	No. of microscopic granulomas[a,b]	No. of NPCs in:		
			Portal tract[a,c]	Parenchyma[a,d]	Central vein area[a,e]
1	8	0	30.6 ± 3.9	4.7 ± 0.7	13.8 ± 2.7
2	7	3.5 ± 4.3[f]	40.2 ± 14.1	9.2 ± 1.4[g]	21.3 ± 3.9[g]
3	6	16.6 ± 4.6[g,i]	57.2 ± 3.9[g,h]	11.6 ± 0.7[g,h]	31.2 ± 8.4[g,h]
4	12	0	28.8 ± 4.2	6.3 ± 0.5	12.6 ± 1.2

[a] All values are means ± standard deviations.
[b] Number of granulomas in a 2-cm^2 section of liver.
[c] Number of nonparenchymal cells (NPCs) in a portal tract of a 30-μm-diameter portal vein.
[d] Number of nonparenchymal cells (NPCs) in an area 30 by 10 μm.
[e] Number of nonparenchymal cells (NPCs) surrounding a central vein with a 30-μm diameter.
[f] Compared with rats with saline-loaded livers, $P < 0.05$.
[g] Compared with rats with saline-loaded livers, $P < 0.001$.
[h] Compared with rats with PG-APS-loaded livers, $P < 0.02$.
[i] Compared with rats with PG-APS-loaded livers, $P < 0.001$.

the numerous grossly evident granulomas that were found on examining the livers of the rats injected i.p. with PG-APS (group 3) and not used as donors. This observation was confirmed histologically since livers from rats injected i.p. with PG-APS (group 3) had 9 to 22 granulomas per 2-cm^2 section of liver tissue whereas livers which were PG-APS-loaded and then transplanted (group 2) had 0 to 9 granulomas per 2-cm^2 section of liver tissue (Table 3).

Architecture was maintained and necrosis and fibrosis were minimal in transplanted saline-injected livers and PG-APS-loaded livers, although some microscopic granulomas were present in the latter group. Nonparenchymal cells (chronic inflammatory cells and Kupffer cells) were increased in PG-APS-loaded livers compared with saline-loaded livers in the portal tracts, around central veins, and in the parenchyma (Table 3). Nonparenchymal cells included mostly liver macrophages (resident Kupffer cells and newly recruited macrophages), although some endothelial cells, fat-storing cells, and lymphocytes were likely counted as well. Nonparenchymal cells were most numerous in livers from rats with the i.p. PG-APS injection (group 3), followed by transplanted PG-APS-loaded livers (group 2), and then transplanted saline-loaded livers (group 1), which were similar to normal rats (Table 3).

DISCUSSION

Spontaneous recurrence of polyarthritis in rats after i.p. injection of PG-APS and reactivation of monoarticular arthritis in PG-APS-injected joints bear considerable resemblance to flares of arthritis in patients with rheumatoid arthritis and arthritis associated with inflammatory bowel disease. To better understand the mechanism of spontaneous recurrences of arthritis following i.p. injection of PG-APS, we determined whether PG-APS-loaded livers could reactivate arthritis in previously injured joints. The main findings of these studies were that (i) transplantation of PG-APS-loaded livers did cause reactivation of arthritis and (ii) PG-APS sequestered within the transplanted livers was disseminated to the blood and spleen. Transplantation of PG-APS-loaded livers caused reactivation of arthritis in joints which had been injured previously by PG-APS. Swelling in contralateral saline-injected joints was not consistently greater in rats which received a PG-APS-loaded liver. Preinjured joints are more sensitive to recurrent inflammation than noninjured joints (5, 25, 29).

The data presented in this study provide direct evidence that bacterial cell wall polymers can be released from depot stores in the liver. In previous studies, 45 days after i.p. PG-APS injection, 1% of the initial dose was recovered in the liver (8, 27). Those results are in close agreement with our present results since livers from rats which received PG-APS i.p. and left intact (group 3) retained 0.8% of the injected material. However, only 0.05% of the original i.p. dose was retained in the liver 56 days after transplantation (group 2). Therefore, in rats injected i.p. and not further manipulated (group 3), PG-APS sequestered in nonhepatic sites (spleen, peritoneal cavity, lymph nodes, or bone marrow) must play an important role in maintaining liver PG-APS levels. This suggests that PG-APS recirculates between extrahepatic sites and the liver. Gradual release of PG-APS from the liver is demonstrated by elevated levels in the plasma and accumulation within the spleen. We hypothesize that there is a constant flux (release and uptake) of PG-APS from sequestered pools into and out of the liver. The mechanism for such release of PG-APS from the liver is unknown but may include transport into bile (14), release of partially degraded PG-APS from Kupffer cells into blood, and release from dying cells. It should be emphasized in the present study that, prior to transplantation, livers were perfused twice so that no freely circulating PG-APS was initially available to the donor.

Our results give insight into the mechanism for spontaneous reactivation of PG-APS-induced arthritis. We had hypothesized that PG-APS released from the transplanted liver would travel to the injured joint and induce reactivation of arthritis. Our data do not support this hypothesis entirely since (i) PG-APS levels in the joint were not increased in rats with reactivation of arthritis and (ii) elevated plasma PG-APS levels did not correlate with reactivation of arthritis.

There are several reasons why PG-APS levels in joints may not be elevated in rats with reactivation of arthritis. Joints were examined only at the end of the experiment, several weeks after peak reactivation of arthritis occurred, so that an earlier accumulation of PG-APS may have been missed. Janusz et al. found a steady decline in PG-APS within joints of rats injected i.p. with PG-APS from 700 to 80 ng/g of tissue 36 days later (8). Our value 56 days after transplantation was approximately 24 ng/g of tissue in group 3, which was not statistically greater than levels in group 2. PG-APS levels in joints may be at the lower range of sensitivity with our ELISA system. The lack of elevated levels of PG-APS within the joints could not be attributed to

anti-PG-PS antibodies or an inhibitor in the ELISA system. Finally, reactivation of arthritis may not be caused by accumulation of PG-APS within the joint.

Circulating PG-APS levels did not temporally correlate precisely with reactivation of arthritis, but rats with a greater total body load of PG-APS had more severe arthritis. Rats in group 3 had a larger PG-APS load than rats in group 2. Sartor et al. (18) found that if the i.p. dose of PG-APS was decreased from 15 to 5 μg of rhamnose/g of body weight, only 40% of rats developed the chronic phase of arthritis and the onset of chronic relapsing arthritis was delayed from 28 to 50 days.

In previous experiments, intravenous injection with small doses of PG-APS induced a flare of arthritis within 6 h which peaked at 3 days (5). However, small amounts of PG-APS consisted of an intravenous bolus of 100 μg of rhamnose (20), which would lead to a calculated immediate plasma level of 5 μg of rhamnose per ml, assuming the blood volume to be 20 ml. The highest plasma levels measured in these experiments was only in the nanogram per milliliter range. Our experiments cannot exclude the possibility that reactivation of arthritis was induced by slow, continuous release of PG-APS from a transplanted liver. Continuous release of PG-APS with sustained blood levels may have entirely different kinetics than that of a single bolus injection. In addition, reactivation of arthritis may require a minimal accumulative dose of PG-APS which is rapidly achieved after intravenous bolus treatment but requires a longer time when release is gradual. The stress of transplantation surgery may have caused release of cortisol and other protective mediators which may have delayed immediate reactivation of arthritis during the first weeks after surgery.

Was reactivation of arthritis secondary to release of cytokines from an inflamed liver? This hypothesis is reasonable since livers from rats in group 3 had more inflammatory cells than those in group 2, and this corresponded to more-severe arthritis in group 3 than group 2. The plasma TNF-α level in rats with PG-APS-loaded livers was slightly elevated compared with that in saline-loaded livers 42 days after transplantation, but the interpretation of this finding is uncertain. At no time point were plasma TNF-α levels increased after i.p. injection of PG-APS despite greater changes in joint diameter and more abnormal liver histology. Liver macrophages are capable of producing large amounts of TNF-α when stimulated with PG-APS (15, 17), and Kupffer cells sequester the majority of PG-APS in the liver (3, 16). However, peak levels of TNF-α after macrophage stimulation are transient and disappear within several hours. In general, one must be cautious when trying to correlate plasma TNF-α levels to events occurring in the joint. TNF-α within joints is probably more relevant since intra-articular injection of TNF-α can reactivate arthritis (24), but serial joint measurements were not possible in this experimental protocol. Other cytokines such as interleukin-1 (20, 25) and platelet-derived growth factor (29), which can also reactivate arthritis in PG-APS-injured joints, could also be important but were not examined.

This animal model may be relevant to human arthritis since absorption of bacterial cell wall polymers such as endotoxins and PG-PS can occur from the intestinal lumen, with enhanced uptake across injured intestinal mucosa (19). These polymers then enter the portal vein and are phagocytosed by macrophages (Kupffer cells) of the liver. The fate of endotoxin has been studied extensively in animals and shows mostly detoxification and elimination (7), but the fate of PG-PS is less well known. Luminal PG-APS is absorbed in increased amounts in rats with small-bowel bacterial overgrowth (10), where it is deposited in the liver. After experimental colonic injury, luminal PG-APS was detected in the liver, spleen, and cardiac blood (19).

These studies begin to investigate the mechanism of reactivation of arthritis induced by bacterial cell wall polymers. Further studies need to examine (i) the role of other cytokines (e.g., interleukin-1 and platelet-derived growth factor) which may originate within the joint or from extra-articular sites and (ii) the role of sensitized T cells in reactivation of arthritis. Our results demonstrate that nonarticular stores of PG-APS, particularly hepatic stores, can be disseminated to the systemic circulation and the reticuloendothelial system. As well, the pool of PG-APS sequestered in nonarticular tissues (liver, spleen, lymph nodes, peritoneal cavity, and bone marrow) can cause reactivation of arthritis. Poorly biodegradable PG-PS originating from the intestinal lumen is an excellent candidate for a bacterial cell wall polymer which could accumulate within a hepatic depot and induce reactivation of arthritis in humans.

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